BIOCHEMICAL EFFECTS OF URETHAN, THIOACETAMIDE AND INTERCELLULAR REACTIONS IN MAMMALIAN CELLS

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Abstract

It has been demonstrated that urethan which is structurally one of the most simple chemical carcinogen interacts with total tissue proteins of lung, liver and kidney tissues of rats and mice. The maximum binding with lung protein was observed 24 hours after the administration of urethan, whereas in the case of liver and kidney tissues maximum protein bound radioactivity was found, at 48 hours and 12 hours respectively. The binding of urethan with proteins gradually declined after reaching the maximum and no radioactivity was found in protein samples at 192 hours, after the urethan administration. As compared to liver and kidney proteins, the extent of urethan binding was more with lung proteins which is in agreement with earlier observations that lung is the most susceptible tissue to urethan carcinogenesis. The data obtained strongly suggest a non-covelent interaction of urethan with tissue proteins. The age of animals has been found to be a determining factor in urethan protein interaction. As compared to older animals (12-6 weeks) the interaction was more in younger animals (3-4 weeks). Since urethan is known to be catabolized much faster in older animals, it is suggested that the length of time,

urethan remains in the animal body may be a critical factor in the initiation of carcinogenesis. The study on intracellular distribution of urethan-C^{14} showed much more concentration of the Carcinogen in mitochondria than nuclei.

The preferential concentration of urethan in mitochondria is suggestive of an important role of mitochondria in carcinogenesis. Mitochondrial swelling and contraction is known to reflect the structural state of mitochondrial membrane and its transport properties. Therefore the effect of thioacetamide and urethan on swelling anc contraction cycle of mitochondria was studied. But this acetamide and urethan induce mitochondrial swelling at 20°C as well as 37°C. The contraction of mitochondria was inhibited at 37°C in presence of both thioacetamide and urethan. Thioacetamide induced swelling has been dependent on the concentration of the carcinogen as well as mitochondria. Since 2,4-dinitrophenol inhibits thioacetamide induced swelling, the process appears to be energy linked. The urethan induced swelling was also dependent on its concentration but it was not as prominent as in the case of thioacetamide. As compared to untreated animals, mitochondria obtained from thioacetamide and urethan induced treated rats failed to respond to inorganic phosphate induced swelling. These results are in agreement with earlier reports where 3-methyl-dimethylaminoazobenzene inhibits swelling of

mitochondria. It is quite possible that both the carcinogens bring about conformational changes in the membrane protein of mitochondria which is responsible for changes in swelling-contraction cycle of mitochondria. However, it needs further experimentation to support this hypothesis.

Since thioacetamide and urethan affected *in vivo* swelling of mitochondria, the *in vivo* effect of these carcinogens on succinate-cytochrome C reductase, a key enzyme of mitochondria was also studied. The results show that single intraperitoneal injections of carcinogenic doses of these compounds bring about a significant decrease in succinate-cytochrome C reductase of rat liver mitochondria.

In order to understand the role of cell contact and intercellular material in determining the biochemical properties in mammalian cells, the effect of cell concentration on the transport and incorporation of labelled amino acids into the proteins of hepatic cells in suspension and Yoshida Sarcoma (ascites) cells was studied. It has been found that the rate of transport and incorporation of labelled amino acids into the proteins decreases when the concentration of hepatic cells in the medium is increased. However, there was no evidence of leakage of amino acids

or accumulation of toxic products in the medium. Similar results were obtained with Yoshida Sarcoma (ascites) cells. These observations suggest that increased frequency of cellular contact is responsible for the reduction in the rate of transport of amino acids in hepatic cells in suspension and Yoshida Sarcoma (ascites) cells. Therefore cell contact could be a factor regulating the metabolic activities in mammalian cells.

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I. ABBREVIATIONS

The following abbreviations have been used without further definition

ADP	adenosine diphosphate
ATP	adenosine triphosphate
CoA	coenzyme A
CPM	counts per minute
Cyt C	cytochrome C
DNA	deoxyribonucleic acid
dpm	disintegrations per minute
gm	gram
hrs	hours
KRP	Krebs - Ringer original Phosphate
M	molar
mc	millicurie
mg	milligram
N	normal
NAD	nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
t-RNA	transfer ribonucleic acid
TCA	trichloroacetic acid
wt	weight

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IV. INTRODUCTION

General - For the last two decades mitochondria have been a subject of intensive biochemical studies for their role in oxidative phosphorylation and localization of citric acid cycle and respiratory chain enzymes. The works of B.Chance, David Green, A.L. Lehninger, E.C.Slater, S. Ochoa, Y. Hateffi, H.A. Lardy and many others have provided a detailed picture of the composition, sequence and the reconstruction of respiratory chain carrier complexes and coupled phosphorylation. With current developments in understanding the basic phenomena of living systems at the molecular level, the knowledge about mitochondria has also acquired new dimensions. It is now established that mitochondria from animal as well as plant cells contain a specific DNA whose buoyant density is different from nuclear DNA (Nass et al., 1965; Granick and Gibor, 1967) and is circular in nature consisting of supercoiled cyclic filaments (Karoon et al., 1966; Dawid et al., 1967; Hudson and Vinograd, 1967; Radloff et al., 1967). Two groups of workers have recently isolated and purified DNA-polymerase from rat liver mitochondria (Kalf and Chik, 1968; Meyer and Simpson, 1968). Circumstantial as well as experimental evidence

has been provided for the presence of mitochondrial DNA dependent RNA polymerase and specific t-RNA (Kalf, 1964; Wintersberger; 1964; Bernett et al., 1967; and Buch and Nass, 1969). Mitochondrial ribosomes have been isolated and characterized from rat liver and Saccharomyces cerevisiae (O' Brien and Kalf, 1967; Stagman et al., 1970). Rat liver mitochondria have also been shown to synthesize protein in vivo and in vitro (Truman, 1963; Kadenbach, 1967). Mitochondria synthesize their messenger RNA and ribosomal RNA (Wintersberger, 1966; Suyama, 1967; Comorosan et al., 1968, Attardi and Attardi, 1968). Mitochondrial DNA has been found to replicate independent of nuclear DNA (Neubert et al., 1965, Reich and Luck, 1966; Smith et al., 1968). These observations clearly present the evidence for an autonomous machinery for DNA, RNA and protein synthesis in mitochondria. Recent evidence that mitochondrial biogenesis is partly under the control of nuclear DNA opens up new avenues for intensive studies on its role in the control mechanism of biochemical reactions in the cell.

Mitochondria from Tumor Tissues - Although considerable amount of work has been done on mitochondria from tumor tissues, little efforts have been made to understand its relevance in the complex phenomenon of

neoplastic transformation.

Tumor mitochondria have been found to be low in NAD, NADH, FAD, CoA, Cyt C, ATP and other adenosine nucleotides (Reid, 1962; Fiala and Fiala, 1967). It has been shown that the activities of ATPase (ATP phosphohydrolase, 3.6.1.4), Aconitase (Citrate(isocitrate)hydrolyase, 4.2.1.3.), Succinate dehydrogenase (Succinate: Cyt. C oxidoreductase, 1.6.2.1.) and Cytochrome Oxidase (Cyt: oxygen oxidoreductase, 1.9.3.1.) are low in hepatoma as compared to normal liver (Schneider, 1946; Aisenberg, 1961; Reid, 1962).

The early concept of Warburg (1923) on impaired respiration and a high rate of glycolysis has recently been supported by the work of Burk et al, (1967). Using a series of tumors, these workers found a direct relationship between the extent of malignancy and anaerobic glycolysis. The suggestion of Neifakh et al (1965), that mitochondria may play an important role in regulating glycolysis by releasing certain glycolysis stimulating factors, is therefore of considerable interest. One of these factors is NAD whose release in normal cells depends upon reversible changes in mitochondrial membrance. Graffi et al (1965) demonstrated significant differences of mitochondrial protein synthesis in vitro in

normal and tumor cells. Compared to normal cells the amino acid incorporation into the mitochondrial proteins of tumor cells was much low. There are other reports also which indicate the absence of certain structural protein in tumor mitochondria (Neifakh et al., 1964; Fiala and Fiala, 1966). In the light of these observations, it is possible that the regulation of glycolysis by mitochondria is lost due to an alteration in the membrane proteins.

Resemblance Between Tumor Cells and Respiratory-Deficient Mutants of Yeast - It is of considerable interest to note the resemblance between tumor cells and respiratory-deficient mutants of yeast. The respiratory-deficient mutants of yeast are known to have an impaired respiration and enhanced glycolysis (Gause, 1967). These mutants of Saccharomyces cerevisiae are not identical but belong to a variety of types depending upon the kind of metabolic impairment. Respiratory-deficient mutants of yeast have been found to lack in Cytochrome Oxidase (Cyt: Oxygen oxidoreductase, 1.9.3.1), Succinic-dehydrogenase-Cyt.b complex and NADH: Cyt C oxidoreductase (1.6.2.1) (Slonimski and Hirsch, 1952). The reduction rates of NADH and NADPH in these mutants have been shown to be extremely low as compared to normal yeast (Kovachevich, 1964). Such

mutants of yeast can be induced by acriflavin (Ephrussi et al., 1949), 5-fluorouracil (Moustacchi and Marcovich, 1963) and carcinogenic agents like 4-nitroquinoline-N-oxide (Mifuchi et al., 1963 and Morita and Mifuchi, 1970) and Styryl 430 (Constantine, 1964).

Recent attempts to investigate the molecular properties of mitochondrial DNA isolated from respiratory-deficient mutants and normal yeast, have indicated a marked difference in their buoyant densities. (Mounoulou et al., 1966). Mehrotra and Mahler (1968) showed that the adenine-thymine content of respiratory-deficient mutant mitochondrial DNA was 90% as compared to 83% for the normal yeast. This alteration according to these workers could make the mitochondrial DNA respiratory-deficient mutant genetically incompetent.

A recent report (Matile and Bahr, 1968) indicates the presence of at least two types of mitochondria in yeast showing different biochemical properties. Similar observations have been made in the case of rat liver where two morphologic types (Bahr and Zeitler, 1962) of mitochondria were found. Electron microscopic studies of tumor mitochondria or mitochondria from carcinogen treated cells as well as from respiratory-deficient mutants of yeast show a great reduction in size and

number (Allard et al., 1952; Schatz et al., 1963 Svoboda and Higginson, 1968).

The above mentioned similarities between respiratory-deficient mutants of yeast and tumor cells suggest a possible microbial model for metabolic organisation of tumor cells. These extremely interesting correlation of biochemical and morphological properties of mitochondria open up several possibilities for work on the role of mitochondria in neoplastic transformation. Two very attractive models highlighting the role of mitochondria in carcinogenesis have been recently proposed (Gause, 1967; Bhargava, 1970).

In the present thesis an attempt has been made to study the early biochemical changes in mitochondria from carcinogen treated animals. The two chemical carcinogens used here are Thioacetamide and Urethan.

Urethan and Thioacetamide Carcinogenesis - Urethan (NH_2-COO-C_2H_5), which is structurally one of the most simple chemical carcinogen, has been shown to induce lung adenomas, hepatomas, mammary tumors, skin papilomas and malignant lymphomas in mice, rats and hamsters and therefore it is regarded as a multipotent carcinogen (Marvish, 1968). On intraperitoneal injection (1 mg/gm body weight) into mice and rats, urethan begins to breakdown

almost immediately and about 90% of the carcinogenic dose is catabolized within 24 hours and only a small amount is excreted in urine (Bryan et al., 1949 Boyland and Rhoden, 1949). A small portion of the administered urethan therefore seems to be responsible for its carcinogenic effects. The mechanism of action of urethan in animal tissues is not yet completely elucidated. However, it is believed that its conversion to N-hydroxy urethan and further acetylation (Boyland and Nery, 1965) or glucosiduronidation (Marvish, 1966) produces a compound which may act as a proximate carcinogen (Nery, 1963).

Thioacetamide (CH_3-CS-NH_2) which is also a structurally simple carcinogen is well-known for its hepatocarcinogenic and other toxic effects in rats and mice. The effects of thioacetamide have been studied histologically and biochemically (Gupta, 1956; Grant and Rees, 1958). Thioacetamide has been found to be rapidly catabolized in rats. Studies with tritium labelled thioacetamide have shown that over 95% of the carcinogenic dose is converted into acetate in 24 hours (Rees et al., 1966). Recently Sapre et al (1969) have reported the metabolic effects of thioacetamide in Swiss mice. Their observations show a decrease in the glycogen and protein contents of liver, while total RNA and DNA contents

of hepatic tissue remained unaltered. These workers have also observed a decrease in the activities of glucose-6-phosphatase (D-glucose-6P phospholydrolase, 3.1.3.9) fructose-1,6-diphosphatase (D-fructose-1, 6diP 1-phosphohydrolase, 3.1.3.11) and phosphoglucomutase (α-D-glucose-1,6 diP: α-D-glucose-1-P phosphotransferase 2.7.5.1) on thioacetamide administration. Glycogen decrease was observed only in the case of liver and not in other tissues, suggesting an organ specific effect of thioacetamide. Grant and Rees (1958) observed a progressive decrease in liver mitochondrial nitrogen content, on treatment with thioacetamide in rats for several weeks. Furthermore, the oxidation rates of l-malate, pyruvate, octanate, citrate, iso-citrate and α-oxoglutarate by mitochondrial suspensions were considerably reduced. The decrease of mitochondrial nitrogen and the alteration in the oxidative capacity of mitochondrial suspension during thioacetamide treatment clearly indicate a direct action of thioacetamide on liver mitochondria.

From the above mentioned properties of urethan and thioacetamide it is evident that these compounds although structurally simple, are potent chemical carcinogens for rats and mice. Since these carcinogens do not require any 'promoter' for the development of tumors and are rapidly catabolized in rodents, one could safely assume that they act

on cellular constituents per se and initiate a process within a short period after their administration which ultimately leads to neoplasia.

Interaction of Chemical Carcinogens with Cellular Proteins - A wide variety of chemical compounds has been shown to be carcinogenic to animals ever since Yamagiwa and Ichikawa (1918) reported the successful production of tumors in rabbits and other experimental animals by repeated painting of coal tar. Chemical compounds capable of initiating the carcinogenic process do not have common structures and also differ in their species and tissue specificities.

Considering the mechanism of induction of neoplasia, it is essential for a chemical carcinogen firstly to penetrate into the cells of the target tissue and secondly to react in a specific way with the cellular constituents. The first factor may depend on the permeability of the tissue to the carcinogen, which may also be one of the factors responsible for its tissue specificity. In the course of interaction of a chemical carcinogen with cellular constituents, its reactions with proteins and nucleic acids seem to be of vital importance in the light of their role in regulating the cellular activities and transfer of genetic properties to daughter cells. Any change or modification in the structure

of these macromolecules as a consequence of their interaction with chemical carcinogens would result in the derangement of control mechanism of cell and might eventually lead to the neoplastic formation.

Chemical carcinogens have been divided into two broad catagories (Farber, 1968), (a) compounds that appear to be active as such and (b) compounds which require metabolic conversion to a more active metabolite to initiate the neoplastic transformation. The first group includes the polycyclic hydrocarbons and β-propiolactone, which are known to effect a variety of cells and tissues in various species. The fact that majority of known metabolites of these compounds are found to be less active or inactive carcinogens support the notion that these chemical carcinogens are active per se and do not require prior conversion to a more active metabolite. In the other group of carcinogens are included the compounds which are subjected to a number of enzymic reactions (Miller and Miller, 1966). In majority of cases these metabolic conversions lead to the formation of compounds that are non-carcinogenic and ultimately detoxicated. However, some metabolic conversions may result in the formation of a compound which may either be equally or more carcinogenic than the parent compound. Such active

metabolites are known as proximate carcinogens and out of these, which finally react with cellular constituents are defined as ultimate carcinogenic metabolites.

It is now known that a number of chemical carcinogens interact with several constituents of cells including protein, RNA and DNA. Interaction of these carcinogens with macromolecules can be either due to formation of weak bonds such as hydrogen bond and electrostatic forces of covalent bonding. These bound derivatives of carcinogens may play an important role in carcinogenesis.

Amino Azo Dyes : Since the original discovery of the covalent interaction of a chemical carcinogen with liver protein by Millers group at Wisconsin, a number of oncogenic chemicals have been found to interact with cell proteins. In their study Miller and Miller (1947) demonstrated a firm binding of N,N-dimethyl-4-aminoazobenzene with liver proteins. Dye binding occured only in liver and an increase in the protein bound dye was found on contineous administration of the dye for several weeks and then it slowly declined. An approximate correlation between the amount of protein bound dye and the susceptibility of the tissue to carcinogenesis by aminoazo dye was also obtained Miller and Miller, 1952; 1953). The hepatic tumors induced by the amino azo dyes

could not show detectable protein bound dye (Miller and Miller, 1947). Sorof et al (1951; 1958; 1963) have shown a preferential binding of aminoazo dyes with the slow h_2 fraction of soluble cytoplasmic proteins from the livers of rats fed with aminoazo dyes (Sorof and Cohen, 1951). They have also shown that the slow h_2 fraction of these proteins increased during the administration of aminoazo dyes and that this fraction was totally missing from the tumors induced by these dyes. Whitcutt et al (1960) have also demonstrated that a major share of 3-methyl-N-dimethyl-4-aminoazobenzene was associated with a single protein fraction isolated by ion-exchange chromatography from the livers of rats fed with this dye. These observations support the early hypothesis of Millers that deletion of an important protein from the cell may induce liver tumors in aminoazo dye fed rats.

As regards the structure of hepatic protein-dye complex, little was known before Millers group isolated 3-methyl mercapto-N-Methyl-4-aminoazobenzene from the livers of rats fed or injected with N-N-dimethyl- or N,methyl-4-aminoazobenzene (Scribner et al., 1965, Miller and Miller, 1966). Their observations suggest the attachement of the dye with protein as a S-methionyl derivative. These results have been confirmed using N-methyl-

4-aminoazo benzene and methionine-S^{35} (Miller and Miller, 1966).

2-Acetyl Amino fluorene (2AAF): Several investigators have studied the binding of 2AAF with cellular proteins in rats using 2-AAF-9-C^{14} or its N-hydroxy derivative and also N^{15}-labelled 2AAF (Miller and Miller, 1955; Dyer and Morris, 1956; Weisburger et al., 1961; Marroquin and Farber, 1965). They have found a correlation between the extent of hepatic protein binding of C^{14}-labelled 2AAF and the susceptibility of the tissue to carcinogenesis (Miller and Miller, 1966). Similar to the studies with aminoazo dyes Millers group has also shown specific binding of esters of 2AAF or its N-hydroxy derivative with sulfur atom of methionine of liver proteins in vivo as well as in vitro (De Bann et al., 1967).

Although the radioactivity is distributed throughout the hepatic soluble protein after the administration of 2AAF-9-C^{14}, Sorof et al (1960; 1965) have observed more localization in the fast h_2 protein fractions. They have also made observations similar to their findings with aminoazo dyes that the h_2 fraction of the liver proteins of rats fed with 2AAF was increased and a marked decrease was found in the h_2 fraction of protein in the hepatic tumors induced by this carcinogen. Furthermore, it has been shown that 2AAF-9-C^{14} does not bind to the proteins of primary hepatic tumors induced by the same carcinogen. (Sorof

et al., 1965). In subsequent studies Freed and Sorof (1966) have shown that purified fractions of h_2 proteins are capable of inhibiting the cell growth in vitro.

2-Napthylamine : Brill and Rodonski (1965) have shown a bound metabolite of 2-naphthylamine in the urinary bladder of dogs which were fed the carcinogen. This study is in agreement with the studies which showed a preferential localization of the radioactivity in urinary bladders of various species which were fed with 2-naphthylamine-8-C^{14} (Henson et al., 1954; Somerville et al., 1956, Goldblatt et al., 1960).

Polycyclic Hydrocarbons : Miller (1951) detected a protein bound derivative of 3,4-benzpyrene in rat liver. Similar observations were made by several other workers using fluorimetric techniques (Moodie et al., 1954; Woodhouse, 1954a, 1954b). Abell and Heidelberger (1962) found preferential binding of hydrocarbons to a slightly basic protein fraction of mouse skin. A correlation between the amount of hydrocarbons bound to protein fraction and the carcinogenisity was also obtained in their studies. Protein binding was also shown with 3,4-benzpyrene and 1,2,5,6 dibenzanthracene on incubation of mouse embryonic cells with these hydrocarbons (Nakajima and Dander, 1964). These observations are of great relevance in the light of the reports of Berwald and Sachs (1963)

and Heidelberger and Ipe (1967) on the malignant transformation of mouse and hamster cells *in vitro* by hydrocarbons.

Ethionine : This chemical carcinogen has also been found to be inactive *per se* and thus needs metabolic conversion to an active metabolite (Stekol, 1963; Farber, 1963). Ethionine is incorporated into proteins in place of methionine. High level of protein binding with ethionie has also been observed by Natori *et al* (1961) in rat liver. Sorof *et al* (1962) have reported some localization of radioactivity in a, g and h_2 soluble proteins. However, the localization was less striking than with the aminoazo dyes or 2 AAF.

Swelling and Contraction of Mitochondria - Mitochondrial swelling was first reported by Cleland (1952) and Raaflaub (1953), who made a detailed study of the volume changes in mitochondria by measuring the turbidity of mitochondrial suspensions and suggested two type of volume changes, (a) a passive change, depending on the osmotic pressure of the suspending medium and (b) an active change linked to respiration. Since then a number of active swelling agents have been discovered. The physiologically occurring swelling agents are inorganic phosphate (Raaflaub, 1953; Hunter and Ford, 1955), thyroxine (Tapley, 1956), reduced and oxidised glutathione

(Neubert and Lehninger, 1962), Ca^{++}(Tapley, 1956), long chain fatty acids (Lehninger and Remmert, 1959), ascorbic acid (Hunter et al., 1959) ferrous ions (Hunter et al., 1963) and four polypeptide hormones like oxytocin, vasopressin, insulin and somatotrophin (Lehninger, 1961, 1964). The non-physiological swelling agents are p-chloromercurobenzoate and iodoacetate (Tapley, 1956; Dickens and Salmony, 1956) metal ions like Zn^{++}, Cu^{++}, Hg^{++} (Tapley, 1956; Hunter et al., 1956) phlorizin (Lehninger and Schneider, 1958), arsenite and sulfite (Hunter et al., 1956, 1959). The Swollen mitochondria may contract again when phosphorylation and respiration are reinstated (Beyer et al., 1955; Price et al., 1956). A rapid and low amplitude shrinkage of mitochondria was demonstrated as a result of addition of ADP in the presence of enough substrates and oxygen (Chance and Packer, 1958; Packer, 1959). However, during large amplitude swelling, phosphorylation and respiration both are inactivated but their restoration only does not shrink the mitochondria. Lehninger (1959) reported the reversal of swelling, by ATP, Mg^{++} and bovine serum albumin. The swelling induced by thyroxine did not require Mg^{++} or Mn^{++} in addition to ATP. The reversal of various types of mitochondrial swelling is not found to be inhibited by 2,4-dinitrophenol and cyanide (Lehninger 1959).

Active large amplitude swelling of mitochondria is accompanied by water and ion uptake and can be produced through three different mechanisms, (a) a mechanism which is linked to respiration through electron transport chain and oxidative phosphorylation. Example of such a mechanism is the swelling of mitochondria induced by inorganic phosphate, throxine, sulfite and arsenite (Tapley, 1956; Hunter et al., 1959). (b) a mode of swelling which is initiated by peroxidation of mitochondrial lipids and is independent of phosphorylating respiration. This type of swelling is induced by ascorbate, glutathione (GSSH or GSH + GSH) and ferrous ions and (c) a third mechanism related to the swelling caused by thiols, disulfides and several peptide hormones.

It is now known that mitochondrial swelling induced by inorganic phosphate or thyroxine requires the generation of a high energy compound (Azzone and Azzi, 1966, Scott and Hunter, 1966). The swelling is accompanied by accummulation of large amounts of cations. The mechanism of energy dependent mitochondrial swelling is not fully known.

Swelling caused by glutathione (GSSH or GSH + GSSH), ascorbate and ferrous ions is independent of phosphorylating respiration because it is not inhibited by 2,4-dinitrophenol, sodium azide or amytal (Hunter

et al., 1959; 1963). Swelling induced by these agents has been found to be related to lipid peroxide formation in mitochondria (Hoffsten et al., 1962 Hunter et al., 1963). However, no lipid peroxidation was found in the case of inorganic phosphate and other agents where mitochondrial swelling is linked to respiration. The question whether lipid peroxidation is the cause or the result of mitochondrial swelling is not yet clear, since in most cases lipid peroxidation and swelling occurs simultaneously. However, it has been suggested that peroxide formation causes the permeability and structural changes in mitochondria during ferrous ion induced swelling (Hunter et al., 1963). The mechanism of peroxide initiating the damage of permeability of mitochondria is not yet known.

In the case of thiols and disulfide (GSH and GSSH) induced mitochondrial swelling, Lehninger (1962) suggested that they possibly react with specific thiol or disulfide groups or both of the mitochondrial membrane . This view is supported by the fact that metals like Hg^{++}, Cu^{++} and p-chloromercurobenzoate as well as iodacetate, all of which are capable of combining with -SH groups, are also swelling agents. These considerations, therefore, suggest that mitochondrial swelling occurs when free

thiol groups in the mitochondria are oxidized or certain mitochondrial disulfide groups are reduced. The externally added thiols could bring about the rupture of disulfide linkage and disulfides would react with membrane thiols to produce a new disulfide bond or in other words the action of thiols and disulfides would bring about conformational changes in the membrance proteins of mitochondria.

The experimental evidence of the above consideration has been provided by Riley and Lehninger (1964). They have observed a large drop in the mitochondrial -SH groups during swelling induced by glutathione (GSSH). The reversal of swelling by ATP, Mg^{++} and bovine serum albumin was accompanied by the restoration of the original thiol content of mitochondrial membrane . No change in mitochondrial -SH groups was observed in the case of mitochondrial swelling induced by Ca^{++}, thyroxine, oleate and glutathione (GSH).

Swelling and contraction cycle of mitochondria has also been studied in carcinogen treated and tumor tissues. It has been found that feeding of 4-dimethylaminoazobenzene and o-aminoazotoluene to rats greatly reduces the swelling ability of liver mitochondria (Clerici and Cudkowicz, 1956; Emmelot and Bos, 1957). Strong impairment of mitochondrial swelling

has been reported from azodye induced hepatoma or from transplanted solid tumors (Emmelot and Bos, 1957; Mululo and Abriqnani, 1957; Miller and Goldfeder, 1965). Inactive azodyes do not bring about such changes in the mitochondria. Feo (1967) has found that tumor mitochondria are initially swollen and therefore could not undergo small or large amplitude swelling to the same extent as normal liver mitochondria. These observations clearly indicate that alteration in the structure of mitochondrial membrances is a characteristic feature of carcinogenesis.

Hepatic Cells in Suspension - Hepatic cells in suspension have been prepared by a number of methods which include mechanical treatment using a gentle homogenization (Harrison, 1953 a, 1953b; Jacob and Bhargava, 1962), treatment with trypsin (Dulbecco and Vogt, 1954; Sandstrom, 1965) and perfusion with chelating agent followed by physical treatment (Anderson, 1953; Eisenberg, 1962). However, most of these methods suffer from a number of drawbacks including low recovery of cells, existence of clumps in final suspension and lack of morphological integrity of the cells. The method described by Jacob and Bhargava (1962) has been most widely used for the preparation of hepatic cells in suspension for biochemical studies, since it has least drawbacks and gives a good recovery of intact parenchymal cells.

Parenchymal cells prepared by this method respire significantly and synthesize protein and RNA (Bhargava and Bhargava, 1962; Jacob and Bhargava, 1965; Ipe and Bhargava, 1965; Reddy, 1969). These cells are known to take up chylomicrons, free or esterified cholesterol and palmitate (Webb and Green, 1966; Higgins and Green, 1966; Higgins 1967). The cells in suspension unlike tissue slices cannot transport orotate, adenine and uracil (Bhargava, 1970). Shanmugam and Bhargava (1966; 1969) have also shown the uptake of exogenously added RNA liver or E.Coli RNA and E.Coli DNA. Comparative studies on the biochemical properties of liver slices and cells in suspension have also been made (Bhargava, 1968). These cells thus provide a good system to study the role of cell contact and intercellular material in determining the intracellular biochemical reactions.

Scope of dissertation - This thesis includes investigations dealing with various aspects of biochemical changes in animal cells on administration of chemical carcinogens and regulation of biochemical reactions as a consequence of cell contact in mammalian cells.

1. Interaction of Urethan-C^{14} with Tissue Proteins in Rats and Mice : It is evident from the work described earlier that the interaction

of chemical carcinogens with cellular proteins is of vital importance in the light of their role as regulators of cellular activity. The main objective of this investigation was to understand the relationship between protein carcinogen interaction and its carcinogenic potency and tissue specificity. To achieve this aim, in vivo interaction of urethan-C^{14} with tissue proteins was studied. Carcinogenic doses of unlabelled urethan (with urethan-C^{14}) were given to rats and mice and protein-urethan-C^{14} interaction was investigated in lung (most susceptible tissue to urethan carcinogenesis), liver and kidney (least susceptible tissue) tissues. Subcellular distribution of urethan-C^{14} has also been studied in view of the earlier reports (Berenblum et al., 1958) that it is preferentially concentrated in mitochondria.

2. Effect of Thioacetamide and Urethan on Swelling and Contraction Cycle and Succinate-cyt. C Reductase of Rat Liver Mitochondria : It is evident from the recent developments in cell biology and biochemical research that mitochondria play a vital role in the regulation of cellular activities. A survey of literature shows that changes in mitochondrial activity in tumor cells were noticed long back but its relevance was not much realized till recent. The following evidence provide rationals to the suggestions that mitochondria might have an important role to play in

the process of carcinogenesis. (Gause, 1967; Bhargava, 1970).

(i) Warburg (1923) observed a high rate of glycolysis and impaired respiration in tumors which has recently been confirmed (Burk et al., 1965, 1967).

(ii) Tumor mitochondria have been found to be low in several enzymes (Reid 1962). The amounts of cytochrome oxidase, cytochrome C, NADH, FAD, CoA, and ATP have also been shown to be much low in cancer cells (Dubois and Potter, 1942; Schneider and Potter, 1943b; Reid, 1962; Fiala and Fiala, 1967).

(iii) Recent evidence, that binding of chemical carcinogens with cellular DNA is directly related to the initiation of carcinogenesis (Hennings et al., 1968, Bates et al., 1968), are of great significance in view of the fact that mitochondria possess their own DNA species.

(iv) Urethan is known to go preferentially into mitochondria (Beremblum, 1958).

(v) The size and number of mitochondria has been found to decrease in tumor cells (Allard et al., 1952; Svoboda and Higginson, 1958).

(vi) Mitochondrial DNA from normal and cancer cells show differences in their physical properties (Clayton and Vinograd, 1969).

(vii) There is a great resemblance between cancer cells and respiratory-deficient mutants of yeast (Gause, 1967). Such mutants may be induced in addition to other agents with chemical carcinogens. The respiratory-deficient mutants of yeast exhibit many similarities with cancer cells including a high rate of glycolysis, impaired respiration and depletion of mitochondrial enzymes. It is now well established that respiratory-deficient mutants of yeast occur as a result of certain abnormalities in mitochondria. Thus these mutants provide an appropriate working model for understanding the role of mitochondria in carcinogenesis.

In view of the above considerations, the effects of thioacetamide and urethan on mitochondrial swelling and contraction (in vitro and in vivo) and Succinate-Cyt C Reductase has been studied. Since thioacetamide and urethan are known to be rapidly removed from the animal body, it seems most likely that initiation of the process which ultimately leads to neoplasia takes place within short period after their administration. Thus the early changes observed in mitochondria after the administration of thioacetamide and urethan would be of great significance.

3. Cell Concentration Effect on the Transport and Incorporation of Labelled Amino Acids in Hepatic Cells in Suspension and Yoshida Sarcoma Cells : Using hepatic cells in suspension, it is possible to study the role of cell contact on the intracellular activities, since the extent and magnitude of cell contact will depend on the concentration of cells in the shaken suspension. In the present study the effect of cell concentration on the transport and incorporation of amino acids in hepatic cells in suspension has been investigated. To know if the phenomenon of cell contact plays a role in determining the biochemical properties of neoplastic cells, similar study has been made with Yoshida Sarcoma (Ascites) cells.

V. EXPERIMENTAL

Materials

Animals - For urethan-protein interaction and cell concentration studies an inbred strain of adult Wistar albino rats were obtained from Nutritional Research Laboratory, Hyderabad. For mitochondrial swelling and enzyme studies an inbred strain of Wistar albino rats were purchased locally. Mice susceptible to urethan carcinogenesis were obtained from Haffkin Institute, Bombay. Wistar albino rats bearing Yoshida Sarcoma (Ascites) cells were obtained from Cancer Research Institute, Bombay. All animals were fed ad libitum on Hindlever Animal Feed.

Chemicals - Special chemicals obtained from commercial sources included: Urethan-(carbony 1-C^{14}) sp. activity, 31.4 mc/mM (Shwartz Bioresearch, U.S.A.); L-Histidine-(ring-2-C^{14}) sp. activity, 35 mc/mM, L-Methionine-(methyl-C^{14}) sp. activity, 29.5 mc/mM, L-Threonine (1-C^{14}) sp. activity, 6.0 mc/ mM, and L-Phenylalanine (1-C^{14}) sp. activity, 170 mc/mM. (Radiochemical Centre, U.K.); Algal Protein Hydrolysate-UL-C^{14}, sp. activity, 54 mc/ mAtom of carbon (Philips-Duphar, Holland); Cytochrome C and ATP (Biochemical Unit, V.P. Chest Inst., Delhi); Thioacetamide and Urethan, (E.Merck, Germany); Folin-Ciocalteu Reagent (BDH, India).

Methods

Isolation of Nuclei and Mitochondria from Lung, Liver and Kidney Tissues- The animals were sacrificed by giving a blow on the head. The tissues were excised, pooled in cold 0.25 M sucrose and homogenized in a pre-cooled all glass or teflon pestle Potter-Elvehjem homogenizer. All manipulations were made at 4-5°C. The homogenate was collected and passed through a 200 mesh sieve to remove connective tissues etc. It was centrifuged at 600 x g in a MSE refrigerated centrifuge. Mitochondria and nuclei were isolated from supernatant and sediment respectively. In case of rat liver mitochondria Janetzki (Model K50) refrigerated centrifuge was used.

(i) Mitochondria : The supernatant was carefully collected and recentrifuged at 600 x g to eliminate any possible nuclei contamination. The resulting supernatant was centrifuged for 30 minutes at 10,000xg. The supernatant was rejected and the sediment containing mitochondria was washed twice with 0.25 M sucrose and finally taken in the required medium.

(ii) Nuclei : The pellet from first centrifugation at 600 x g, containing whole cells, cell debris and nuclei was dispersed mechanically at a very low speed in a teflon pestle homogenizer which contained 2.4 M sucrose in 1.5×10^{-3} M $CaCl_2$. After centrifugation at 40,000 x g for

60 minutes in a SW 25.1 rotor of a Spinco (model L) Preparative Ultracentrifuge, the nuclei formed a thick pellet at the bottom of the centrifuge tube. The nuclei pellet was re-dispersed in 2.4 M sucrose containing 1.5×10^{-3} M $CaCl_2$ and again centrifuged at 40,000 x g for 60 minutes. To ascertain the purity of the nuclei preparation, a sample of the nuclei pellet was checked under a phase cotrast microscope (x 80). If necessary it was resuspended and recentrifuged at 40,000 x g for 60 minutes.

Measurement of Swelling and Contraction of Mitochondria - The swelling and contraction of rat liver mitochondria was measured according to the method of Lehninger (1959) by following the decrease and increase in absorbance at 520 mu respectively in a Bausch and Lomb Spectronic 20 spectrophotometer. For inorganic phosphate, thioacetamide and urethan induced swelling, the temperature was maintained at 20-22°C, whereas for spontaneous swelling experiments it was kept at 37°C. The protein content of mitochondria was estimated by Lowry (1951) method.

Assay of Succinate-Cytochrome C Reductase - Using succinic acid and cytochrome C as electron donor and acceptor respectively, mitochondrial succinate-cytochrome C reductase was assayed manometrically (Schneider and Potter 1943).

Preparation of Hepatic Cells in Suspension - It was prepared according to the method of Jacob and Bhargava (1962). The method consists of perfusion

of the liver with 30-50 ml of cold 2.7 x 10^{-2} M sodium citrate in calcium-free Lock's solution (Dawson et al., 1959), followed by dispersion in 0.25 M sucrose in a specially designed tissue disperser which is a modified Potter-Elvehjem homogenizer having a soft rubber pestle. The dispersate was filtered through a 200 mesh sieve and centrifuged in a MSE refrigerated centrifuge for a minutes at 200 x g. The sediment of the single parenchymal cells was resuspended in cold Ca^{++}-free KRP buffer(Dawson et al.,1959

Preparation of Yoshida Sarcoma (ascites) Cells - Ascites cells drawn out withthe help of a syringe from the peritoneal cavity of rats were collected in cold normal saline solution. They were spun down at a low speed in a refrigerated centrifuge and supernatant containing blood cells was discarded. The cells were repeatedly washed with cold normal saline till all the blood cells were removed and a pure preparation of ascites cells was obtained. These cells were once again washed with Ca^{++}-free KRP buffer and suspended in the same medium.

Cell Count - The concentration (number of cells/ml) of parenchymal cells and Yoshida sarcoma cells was determined on a haemocytometer (100 u deep having a graduated area of 9 mm^2 divided into 9 major squares each with an area of 1 mm^2) under phase contrast microscope (x 80).

Isolation and Estimation of Protein and Total Free Amino Acids in Hepatic Cells in Suspension and Yoshida Sarcoma Cells - At the end of incubation period the samples were chilled in ice, centrifuged and the supernatant rejected. The pellet was repeatedly washed with cold Ca^{++}-free KRP buffer to remove all the radioactivity in the medium. The washings were monitered for their radioactivity till no counts were detected. For complete precipitation of proteins the cells were precipitated by 10% TCA (w/v) and kept in cold for 20 minutes. The precipitate was centrifuged and the supernatant was kept for free amino acid estimation. The precipitate was washed twice with 5% TCA. These washings were combined with the first one for free amino acid estimation. The precipitate was then heated in 5% TCA for 30 minutes at 90°C to hydrolyse the nucleic acids and washed twice with 5% TCA again. Lipids were removed by washing twice with 50% alcohol, twice with 100% alcohol. twice with alcohol-ether (3:1) and finally twice with ether. The protein residue obtained was used for estimation of radioactivity.

The pooled TCA washing collected earlier was shaken with ether till all TCA was removed as checked by pH paper. The aquous solution which now contained only amino acids and other low molecular weight subst-

ances was passed through a column of Zeo-karb 225 resin (150-200 mesh, hydrogen form, 0.2 x 12 cm). Under these conditions only amino acids are known to be retained by the resin. The amino acids were eluted by 2 N aquous ammonia. The eluent (10-20 ml) was evaporated to dryness under vacuum. The residue was dissolved in 1 ml of water and aliquotes from this were taken for the estimation of radioactivity and amino acids. This method is essentially the same as followed by Bhargawa et al (1959).

Estimation of Free Amino Acids and Protein - Free amino acids were estimated by Ninhydrin photometric method of Moor and Stein (1948) taking leucine as standard. Protein was estimated either by Lowry's (1951) method or expressed as dry weight.

Paper Chromatography of Amino Acids - Free amino acids obtained from hepatic cells were separated by two-dimentional chromatographic method using n-butanol-aceticacid-water (4:1:5) and phenol-ammonia (200:1). The spots were developed with Ninhydrin, cut into pieces and the amino acids eluted with 60% alcohol. Radioactivity was determined for each amino acid spot.

Radioactivity Assay - The total tissue proteins obtained as described above were plated as a slurry in 30% alcohol on 2 sq. cm. aluminium planchets. The planchets were dried under vacuum. The radioactivity was measured in a

Philips Geiger- Muller counter of a Tracerlab and window Geiger Muller counter. All samples were counted to a statistical significance of 5% as recommended by Calvin et al (1949). Appropriate correction for self-absorption was made wherever necessary.

Calculations of Dry Weight - Dry weight of hepatic cells in suspension were calculated according to the unpublished results of Hussain, L.F., Gopinath B and Bhargava, P.M. where 1 x 10^6 hepatic cells of 4 month old rat weighing about 150-160 gms are equivalent to 0.82 mg dry weight. The dry weight of perfused and unperfused rat liver slices were calculated according to Ipe, P.T., Bhargava, P.M. and Taskar, A.D. (1965).

VI. RESULTS

1. INTERACTION OF URETHAN-C^{14} WITH TISSUE PROTEINS OF RATS AND MICE

a. Interaction of Urethan-C^{14} with Total Cellular Proteins of Lung, Liver and Kidney Tissues of Rats - Significant amount of radioactivity was found in protein isolated from lung, liver and kidney tissues of rats after 24 hours of intraperitoneal injection of urethan-C^{14}. Radioactivity in proteins was much less after 48 hours (Table I.)

b. Interaction of Urethan-C^{14} with Total Cellular Proteins of Lung, Liver and Kidney Tissues of Young Mice - Binding of urethan was studied with total cellular proteins of lung, liver and kidney tissues of mice at different time intervals after an intraperitoneal injection of urethan-C^{14}. As shown in Figure 1 the maximum binding of urethan was found in lung protein at 24 hours. The extent of binding of urethan with liver and kidney tissues was almost the same. The maximum binding of urethan-C^{14} with protein of kidney tissue takes place at 12 hours whereas it was maximum for liver protein at 48 hours after the administration of urethan. In all cases the protein bound radioacitity was found to decline after reaching the maximum and no binding of urethan with proteins was observed after a week (Table II, Fig.1).

T A B L E - I

Interaction of Urethan-C^{14} with Total Cellular Proteins of Lung, Liver and Kidney Tissues of Rats

Rats in groups of two were given single intraperitoneal injections of 0.1 mg of unlabelled urethan per gm. body weight containing 50 µc of urethan-C^{14}. Animals were killed at 24 hours and 48 hours after the administration of urethan and tissues were removed. Protein was isolated by TCA method as described in methods. The values in parentheses represent the specific activities of protein after heating the samples for 18 hours at 110-120°C. All samples were counted to a statistical significance of 5%.

Tissue	Specific activity of Protein (CPM/mg dry wt. Protein)	
	24 hours	48 hours
Lung	2174 (582)	27
Liver	925 (94)	17
Kidney	2354 (371)	0

T A B L E - II

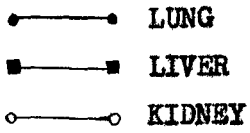
Interaction of Urethan-C^{14} with Total Cellular Proteins of Lung Liver and Kidney Tissues of Young Mice

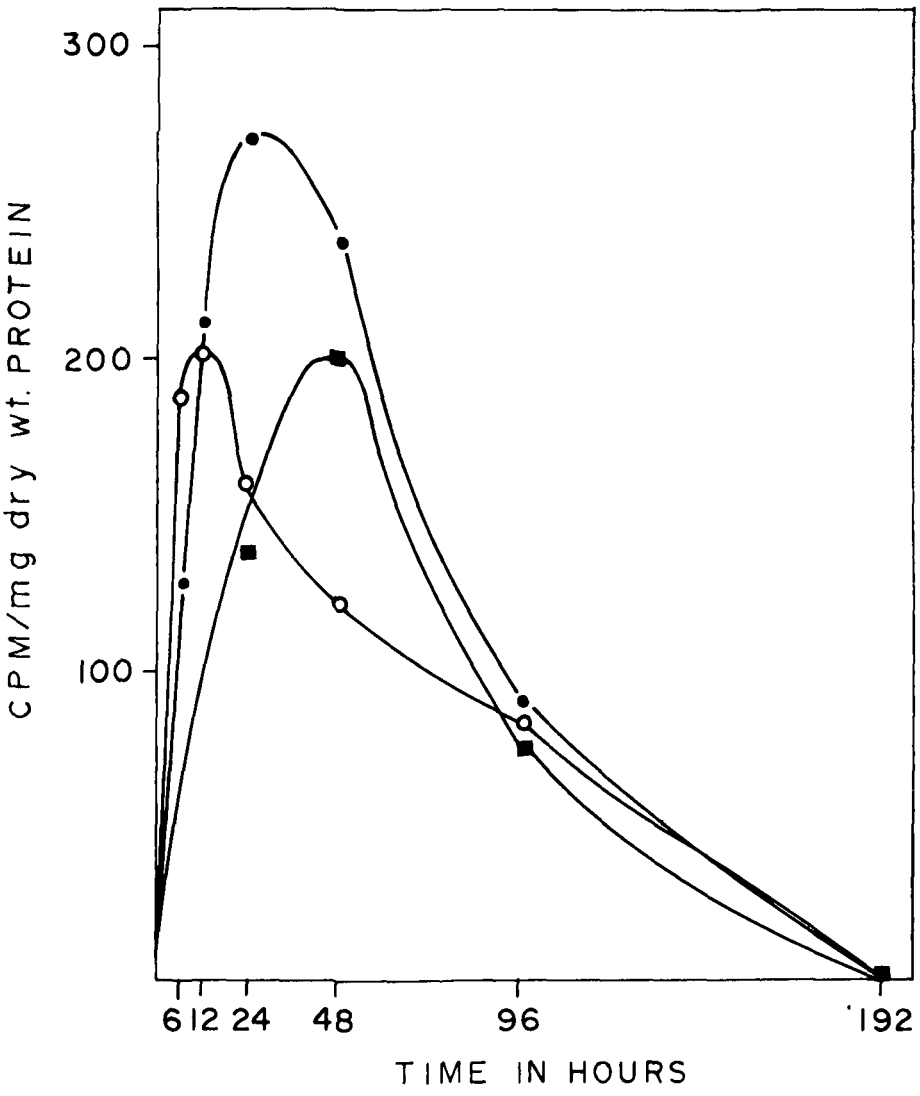
Eighteen young male mice, 3-4 weeks old (15 gm) were given a single intraperitoneal injection of 15 mg of unlabelled urethan containing 10 μ c of urethan-C^{14}. Three mice were killed each at 6, 12, 24, 48, 96 and 192 hours after the administration of urethan. The proteins were isolated by TCA method. The values in parentheses represent the specific activities of protein after heating the samples for 18 hours at 110-120°C. All samples were counted to a statistical significance of 5%.

Tissue	Specific Activity of Protein (CPM/mg dry wt. Protein)					
	6 hours	12 hours	24 hours	48 hours	96 hours	192 hours
Lung	127(24)	211(32)	268(26)	240(22)	90(5)	2 (0)
Liver	136(14)	99(16)	134(7)	201(8)	96(1)	3 (0)
Kidney	186(21)	200(18)	159(9)	119(7)	87(8)	0 (0)

Figure 1. Effect of time on the interaction of urethan-C^{14} with tissue proteins of mice

The data of Table II has been plotted as a function of time




300
200
100
CPM/mg dry wt. PROTEIN
6 12 24 48 96 192
TIME IN HOURS

c. Effect of Heating on Protein bound Urethan-C^{14} - As shown in Table II about 90% of the radioactivity in the protein bound urethan-C^{14} samples was lost as a result of heating at 100-120°C for 18 hours, similar results were obtained in the case of protein bound urethan-C^{14} isolated from the tissues of rats (Table I).

d. Interaction of Urethan-C^{14} with Total Cellular Proteins of Lung, Liver and Kidney Tissues of Older Mice - In order to see if age of the animal has any relation with the urethan protein interaction, 12-16 weeks old mice were treated similarly. Very little binding of urethan was found with cellular proteins (Table III).

e. Distribution of Urethan-C^{14} into Nuclei and Mitochondria of Lung, Liver and Kidney Tissues of Mice - Due to the elastic nature of lung cells, there was difficulty in homogenizing the lung tissues and, therefore, nuclei and mitochondria could not be isolated in much purified form from it as compared to liver and kidney tissues. However, the data presented in Table IV indicate that urethan was concentrated more in mitochondria than in nuclei of liver and kidney tissues.

T A B L E - III

Interaction of Urethan-C^{14} with Total Cellular Proteins of Lung, Liver and Kidney Tissues of Older Mice

Six old male mice, 12-16 weeks, (20 gm) were given single intraperitoneal injections of 30 mg of unlabelled urethan containing 15 uc of urethan-C^{14}. One mouse was killed each time at 5, 12, 24, 48, 96 and 192 hours after the administration of urethan. Tissues were removed and proteins were isolated by TCA method. All samples were counted for 30 minutes to a statistical significance of 5%.

Tissue	Specific Activity of Protein (CPM/mg dry wt. Protein)					
	5 hours	12 hours	24 hours	48 hours	96 hours	192 hours
Lung	1	2	23	4	13	5
Liver	6	10	30	8	12	10
Kidney	4	18	31	4	5	0

T A B L E - IV

Distribution of Urethan-C^{14} into Nuclei and Mitochondria of Lung, Liver and Kidney Tissues of Mice

Four male mice 12-16 weeks old (30 gm) were given single intraperitoneal injection of 30 mgs of unlabelled urethan containing 15 uc of urethan-C^{14}. The animals were killed at 12 hours after the administration of urethan. Tissues were pooled together and homogenized in 0.25 M sucrose. Nuclei and Mitochondria were isolated as described in materials and method. The proteins of nuclei and mitochondria were estimated by Lowry method. All samples were counted to a statistical significance of 5%.

Tissue	Nuclei (CPM/mg protein)	Mitochondria (CPM/mg protein)
Lung	333	191
Liver	106	630
Kidney	275	340

2. EFFECT OF THIOACETAMIDE AND URETHAN ON SWELLING AND CONTRACTION CYCLE AND SUCCINATE-CYT.C REDUCTASE OF RAT LIVER MITOCHONDRIA

a. Effect of Thioacetamide and Urethan on Spontaneous Swelling - Spontaneous swelling of rat liver mitochondria was found to be stimulated to the extent of 28.2% and 24.3% by thioacetamide and urethan respectively (Table V). The reversal of swelling or contraction of mitochondria by ATP, $MgCl_2$ and bovine serum albumin was inhibited by 23.3% and 23.2% respectively (Fig.2A and B).

b. Thioacetamide Induced Mitochondrial Swelling - Thioacetamide has also been found to induce mitochondrial swellin at 20°C.

(i) Effect of Thioacetamide Concentration : Induction of mitochondrial swelling was studied at various concentration. As shown in Fig.3A, maximum swelling was observed with 20mM thioacetamide. When mitochondria were kept in cold (0-5°C) for 2 hours or more, the induction of swelling was inhibited to the extent of 60-66%.

(ii) Effect of Mitochondrial Concentration : A linear relationship between the concentration of mitochondria (taken as the amount of mitochondrial protein) and the extent of swelling induced by thioacetamide has been found (Fig.3B).

TABLE - V

In Vitro Effect of Thioacetamide and Urethan on the Spontaneous Swelling and Contraction of Rat Liver Mitochondria

The incubation mixture in total volume of 5.0 ml finally contained 0.75 M KCl-0.02 M Tris-HCl, pH 7.4, 5mM thioacetamide or urethan and mitochondria as indicated. Swelling of mitochondria was studied by following the decrease in absorbance at 520 mu at 37°C. The contraction of mitochondria was studied by addition of 0.1 ml of a solution containing 25 umoles ATP, 15 umoles $MgCl_2$ and 200 ugm bovine serum albumin adjusted to pH 7.4 after maximum swelling was achieved.

Carcinogen	Protein concentration (in mg)	Percent Stimulation of Mitochondrial Swelling	Percent Inhibition of Mitochondrial contractio
Thioacetamide	3.51 ± 0.7	28.2 ± 9	23.3 ± 3
Urethan	3.12 ± 0.8	24.3 ± 4	23.2 ± 9

$$\% \text{ Stimulation} = \frac{\text{Total decrease in O.D.(Treated - Control)}}{\text{Total decrease in O.D in Control}} \times 100$$

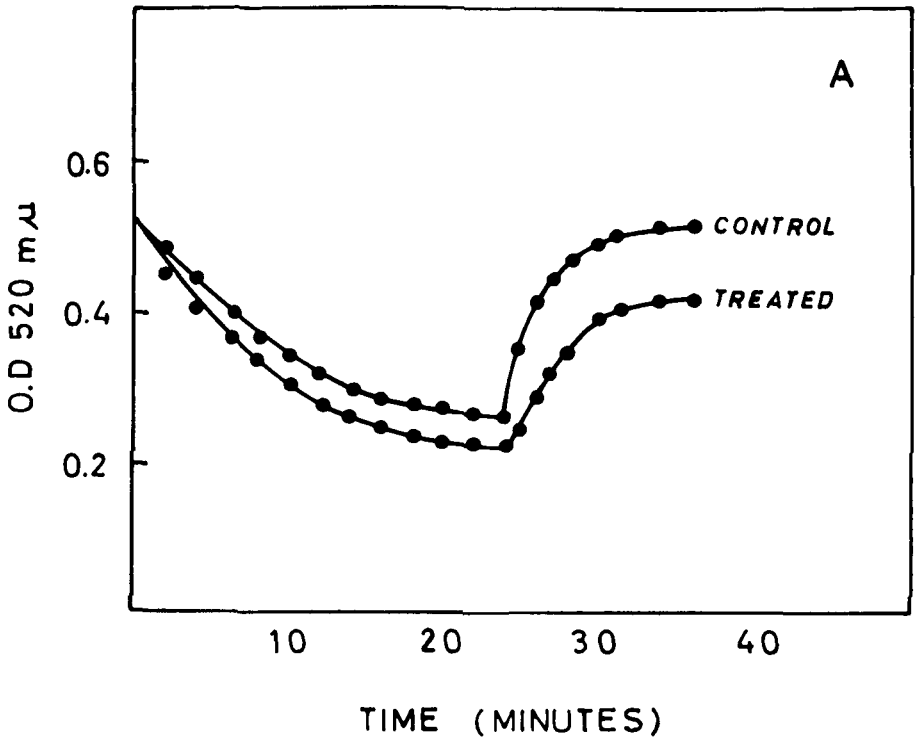
$$\% \text{ Inhibition} = \frac{\text{Total increase in O.D.(Control - Treated)}}{\text{Total increase in O.D. in Control}} \times 100$$

Figure 2. Effect of thioacetamide and urethan on spontaneous swelling and contraction of rat liver mitochondria

The incubation mixture was same as described for Table V.

A ... Thioacetamide

B ... Urethan


A
0.6
0.4
0.2
O.D 520 mμ
CONTROL
TREATED
10
20
30
40
TIME (MINUTES)

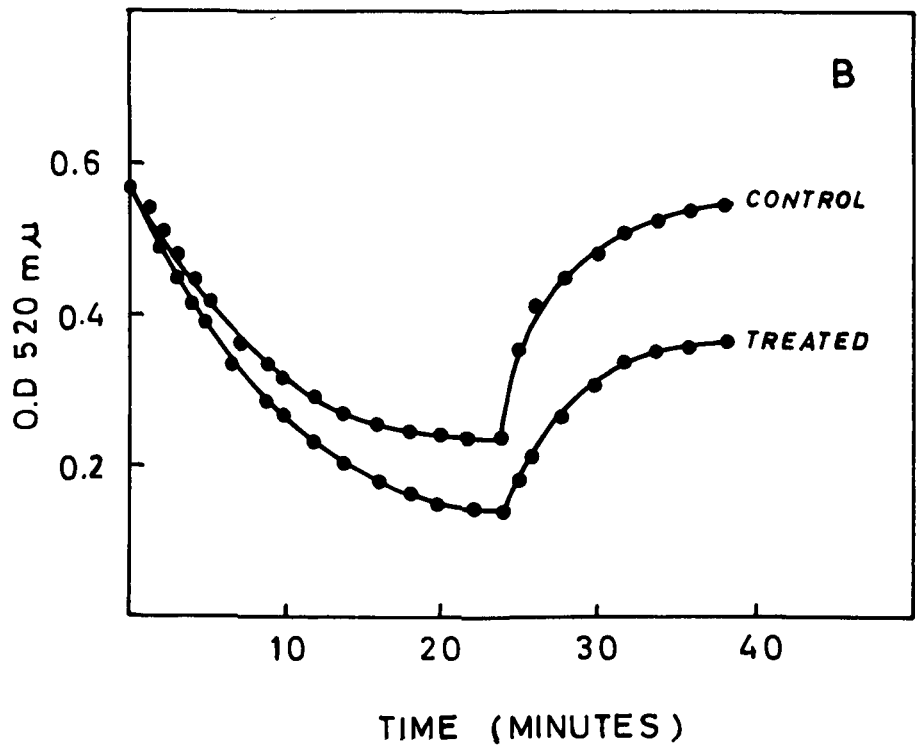

B
0.6
0.4
0.2
O.D 520 mμ
CONTROL
TREATED
10
20
30
40
TIME (MINUTES)

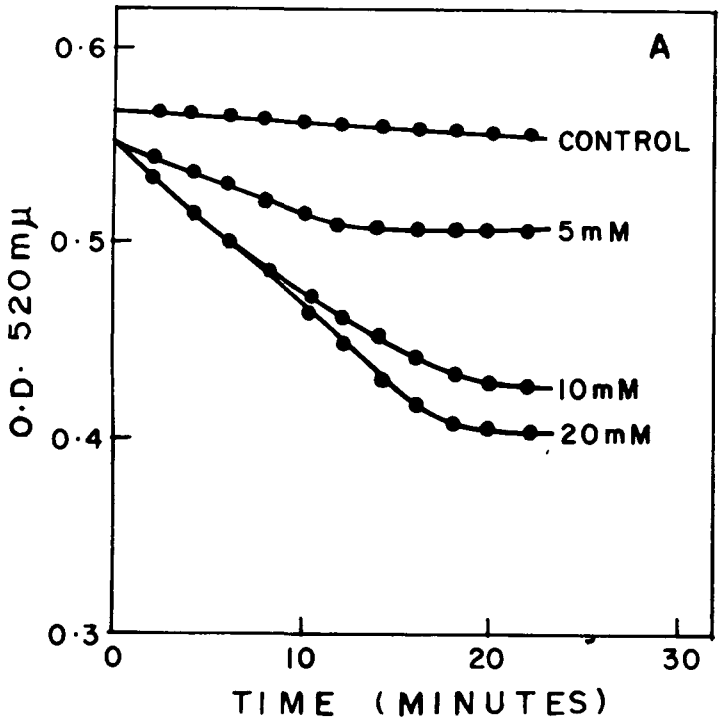
Figure 3.A. Thioacetamide induced swelling of rat liver mitochondria

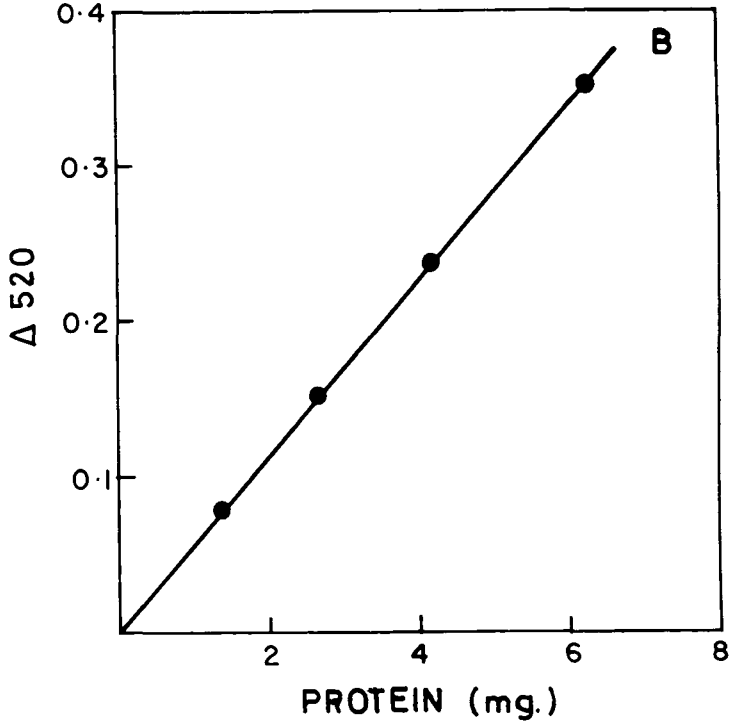
The incubation mixture in a total volume of 5.0 ml finally contained 0.75 M KCl-0.02 M Tris-HCl, pH 7.4, 3.8 mg mitochondrial protein and varying final concentrations of thioacetamide as indicated. Mitochondrial swelling was followed by observing the decrease in absorbance at 520 m μ at 20-22°C. A parallel control without thioacetamide was also run

B. Effect of mitochondrial concentration on thioacetamide induced mitochondrial swelling

The incubation mixture was similar as above except the final concentration of thioacetamide (20mM) and varying concentrations of mitochondria as indicated.

Δ 520 = maximum decrease in absorbance at 520 m μ


A
0·6
0·5
0·4
0·3
O·D· 520 mμ
CONTROL
5 mM
10 mM
20 mM
0
10
20
30
TIME (MINUTES)


B
0·4
0·3
0·2
0·1
Δ 520
2
4
6
8
PROTEIN (mg.)

(iii) Effect of Metabolic inhibitors : As shown in Figure 4, 75% of the mitochondrial swelling induced by thioacetamide was inhibited by 2,4-dinitrophenol whereas sodium cyanide did not have any effect on the swelling.

c. Urethan Induced Mitochondrial Swelling - Induction of mitochondrial swelling at different concentration of urethan was studied. The swelling in this case was not as prominent as with thioacetamide (Fig.5).

d. In Vivo Effect of Thioacetamide and Urethan on Mitochondrial Swelling - Twenty four hours prior to killing rats were given an intraperitoneal injection of 0.25 mg thioacetamide/gm body weight and 1.0 mg urethan/gm body weight. Liver mitochondria isolated from these animals did not response to inorganic phosphate induced swelling to the same extent as mitochondria from untreated animals. Both carcinogens caused 30% inhibition of mitochondrial swelling (Fig.6).

e. Effect of Thioacetamide on Succinate Cytochrome-C Reductase in Isolated Rat Liver Mitochondria - As shown in Table VI, Fig.7 the activity of succinate-Cyt.C reductase in rat liver mitochondria has been found to decrease considerably at 3 hours after the administration of thioacetamide. It further decreased reaching a minimum of 28 per cent activity at 48 hours. The enzyme activity was then found to increase.

Figure 4. Effect of 2,4-dinitrophenol on thioacetamide induced swelling of rat liver mitochondria

The control tube in a total volume of 5.0 ml finally contained 0.75 M KCL-0.02 M Tris-HCl, pH 7.4 and 3.6 mg mitochondrial protein. 100 μ moles thioacetamide and 0.5 μ moles 2,4-dinitrophenol were added as indicated. Swelling of mitochondria was studied by following the decrease in absorbance at 520 m μ at 20-22°C.

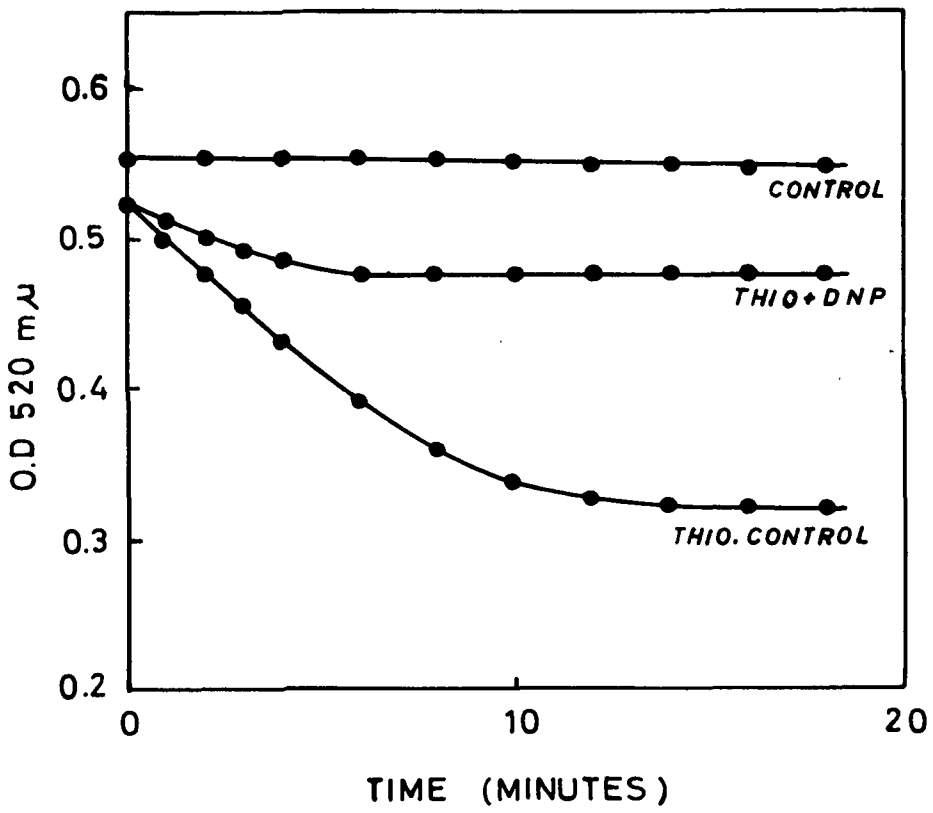

0.6
0.5
0.4
0.3
0.2
O.D 520 mμ
CONTROL
THIO+DNP
THIO. CONTROL
0
10
20
TIME (MINUTES)

Figure 5. Urethan induced swelling of rat liver mitochindria

The incubation mixture in a total volume of 5.0 ml finally contained 0.75 M KCl-0.02 M Tris-HCl, pH 7.4, 3.6 mg mitochondrial protein and varying final concentrations of urethan as indicated. Mitochondrial swelling was studied by following the decrease in absorbance at 520 m μ at 20-22°C. A parallel control without urethan was also run

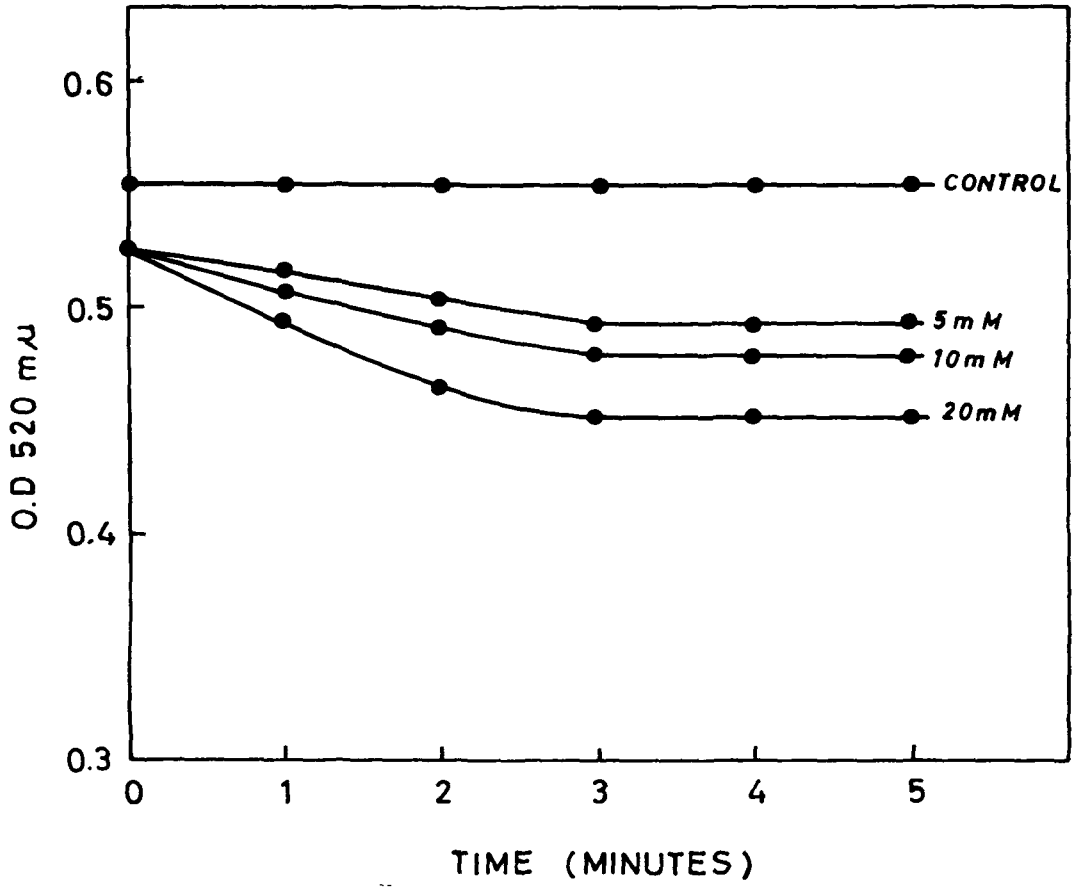
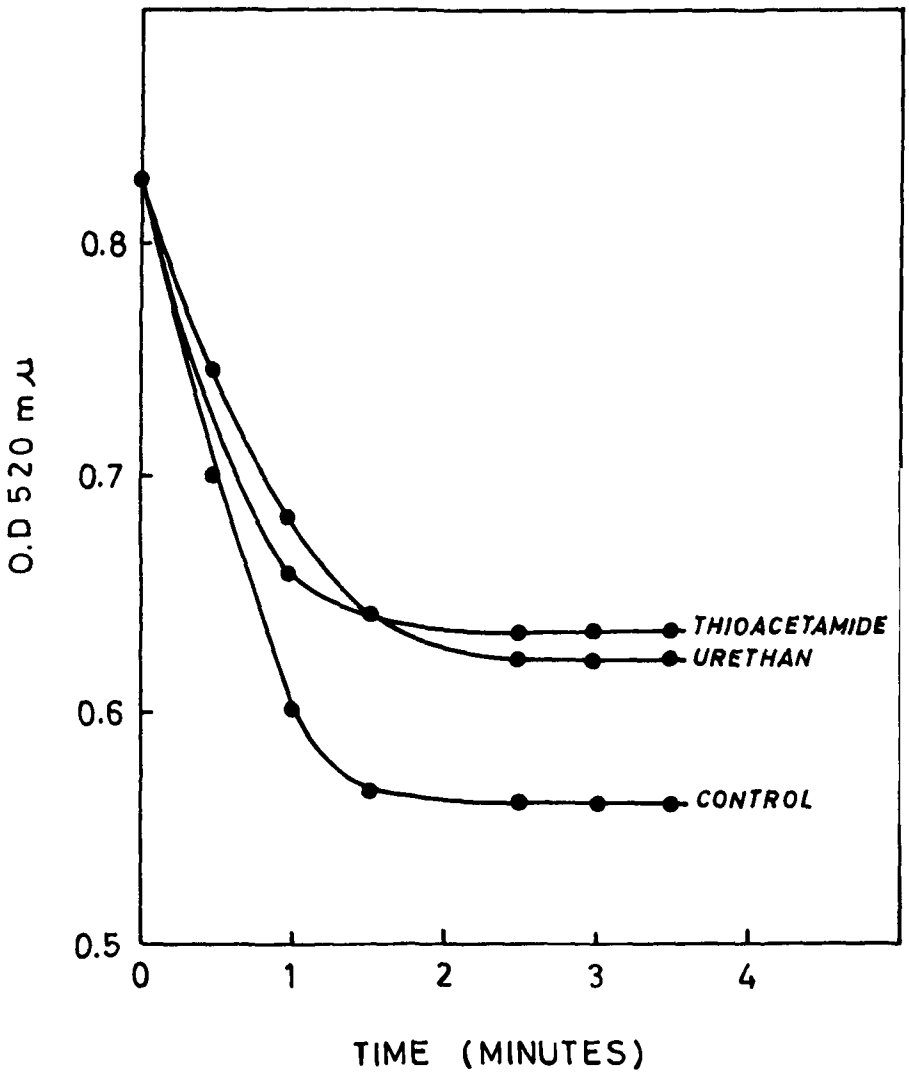

0.6
0.5
0.4
0.3
O.D 520 mμ
0
1
2
3
4
5
TIME (MINUTES)
CONTROL
5 m M
10 m M
20 m M

Figure 6. In vivo effect of thioacetamide and urethan on swelling of rat liver mitochondria

The incubation mixture in a total volume of 5.0 ml finally contained 0.75 M KCl-0.02 M Tris-HCl, pH 7.4, 0.1 M inorganic phosphate and 5.6 mg mitochondria (Protein) from thioacetamide and urethan treated animals. The control tube contained same amount of mitochondria from untreated animals. Animals were prepared as described in text. Swelling of mitochondria was studied by following the decrease in absorbance at 520 m μ at 20-22°C.


0.8
0.7
0.6
0.5
O.D 520 mμ
0
1
2
3
4
TIME (MINUTES)
THIOACETAMIDE
URETHAN
CONTROL

T A B L E - VI

Effect of Thioacetamide on Succinate-Cytochrome C Reductase of Rat Liver Mitochondria

Rats in groups of 5 were given intraperitoneal injections of 0.25 mg thioacetamide per gm body weight. Animals were killed at 3, 24, 48, 96 and 192 hours after the administration of urethan. Mitochondria were isolated from liver as described in methods. Separate control were taken for different time points.

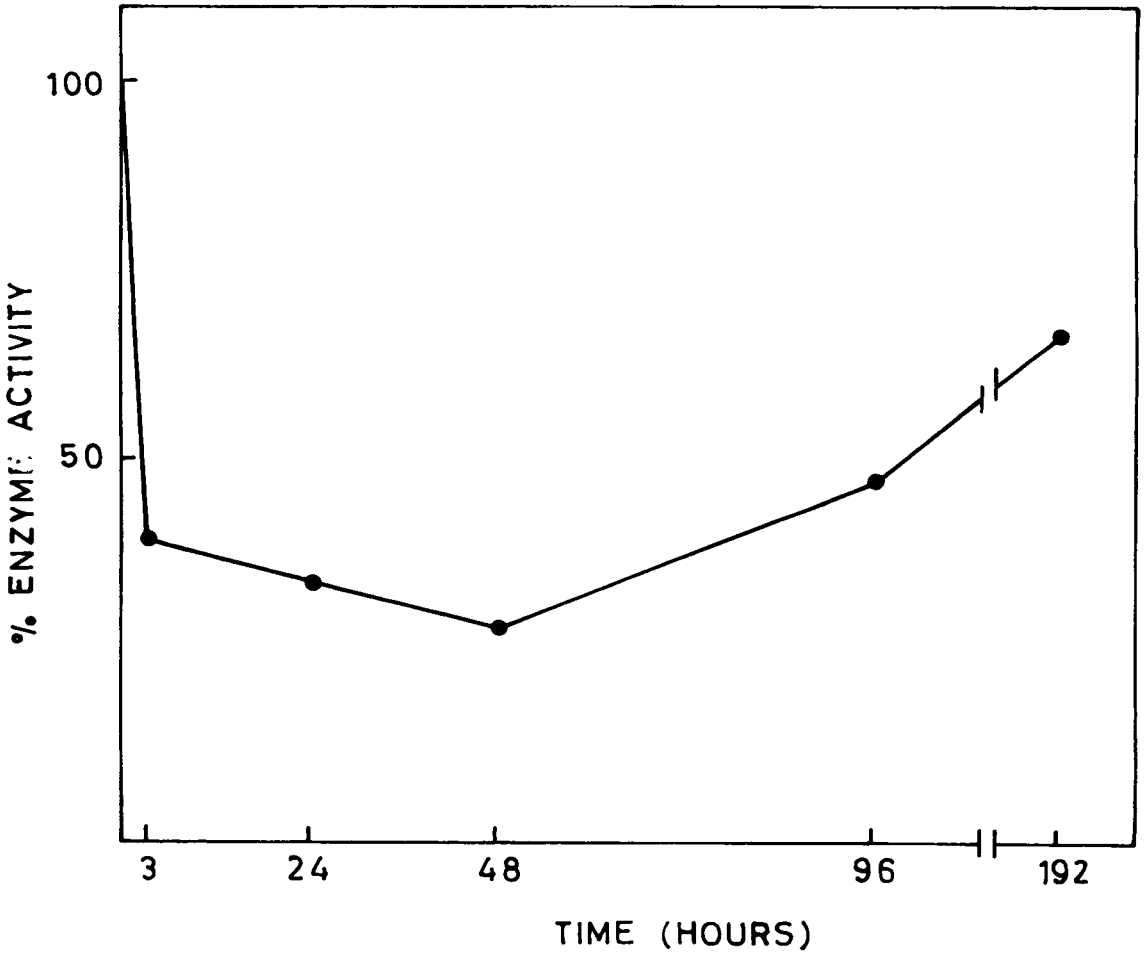
Mitochondria were incubated in Wrburg flasks at 37°C. The incubation mixture in a total volume of 3.0 ml contained 100 µmoles sodium phosphate buffer, pH 7.4; succinate, 150 µmoles; cytochrome C(red), 4×10^{-5} µmoles; $CaCl_2$, 1.2 µmoles and $AlCl_3$, 1.2 µmoles. The reaction was started by tipping 0.4 ml Cyt. C from the side arm. The centre cup contained 0.2 ml of 2N NaOH. The enzyme activities were calculated as average $\mu l O_2$/hr/mg of mitochondrial protein. The average enzyme activity in untreated (control) animals for different time points was 183±8 $\mu l O_2$/hr/mg of mitochondrial protein.

Time (Hrs.)	Enzyme Acitity	Percent Enzyme Activity
3	71± 4	39
24	62± 4	34
48	52± 3	28
96	86± 6	47
192	121± 2	66

$$\text{Percent enzyme activity} = \frac{\text{Enzyme activity in treated animals}}{\text{Enzyme activity in untreated animals}} \times 100$$

Figure 7. Effect of thioacetamide on succinate-cytochrome C reductase of rat liver mitochondria

The data of Table VI has been plotted as a function of time


100
50
% ENZYME ACTIVITY
3
24
48
96
192
TIME (HOURS)

However, the activity was only 66 per cent of the control (untreated) mitochondria at 192 hours after the adminstration of the carcinogen.

f. Effect of Urethan on Succinate-Cyt.C Reductase in Isolated Rat Liver Mitochondria - The activity of succinate-cyt c Reductase was only 34 per cent of the initial activity in mitochondria isolated from animals killed after 3 hours of urethan administration. The enzyme activity was only 60 per cent after 48 hours of urethan administration. There was no appreciable change in enzyme activity even after 192 hours (Table VII, Figure 8).

3. CELL CONCENTRATION EFFECT ON THE TRANSPORT AND INCORPORATION OF LABELLED AMINO ACIDS IN HEPATIC CELLS IN SUSPENSION AND YOSHIDA SARCOMA CELLS

a. Effect of Cell Concentration on the Transport and Incorporation of l-Histidine-C^{14} and l-Methionine-C^{14} in the Hepatic Cells in Suspensions - As shown in Table VIII, IX and X and Figure 9(A, B and C), it was found that the specific activity of proteins and the specific activity of free amino acid pool decreases when the concentration of hepatic cells in the medium is increased. The range of concentration of cells used in different experiments was from 0.34×10^6 cells/ml to 6.0×10^6/ml. The radioactivity was further checked in the Histidine spot on the chromatogram. A decrease in the radioactivity was observed when the concentration of cells was increased as shown in Table IX and Figure 9(c).

T A B L E - VII

Effect of Urethan on Succinate-Cytochrome C Reductase in Rat Liver Mitochondria

Rate in groups of 5 were given intraperitoneal injections of 1.0 mg of urethan per gm body weight. Animals were killed at 3, 24, 48, 96 and 192 hours after the administration of urethan. Mitochondria were isolated from liver as described in methods. Separate controls were taken for different time points.

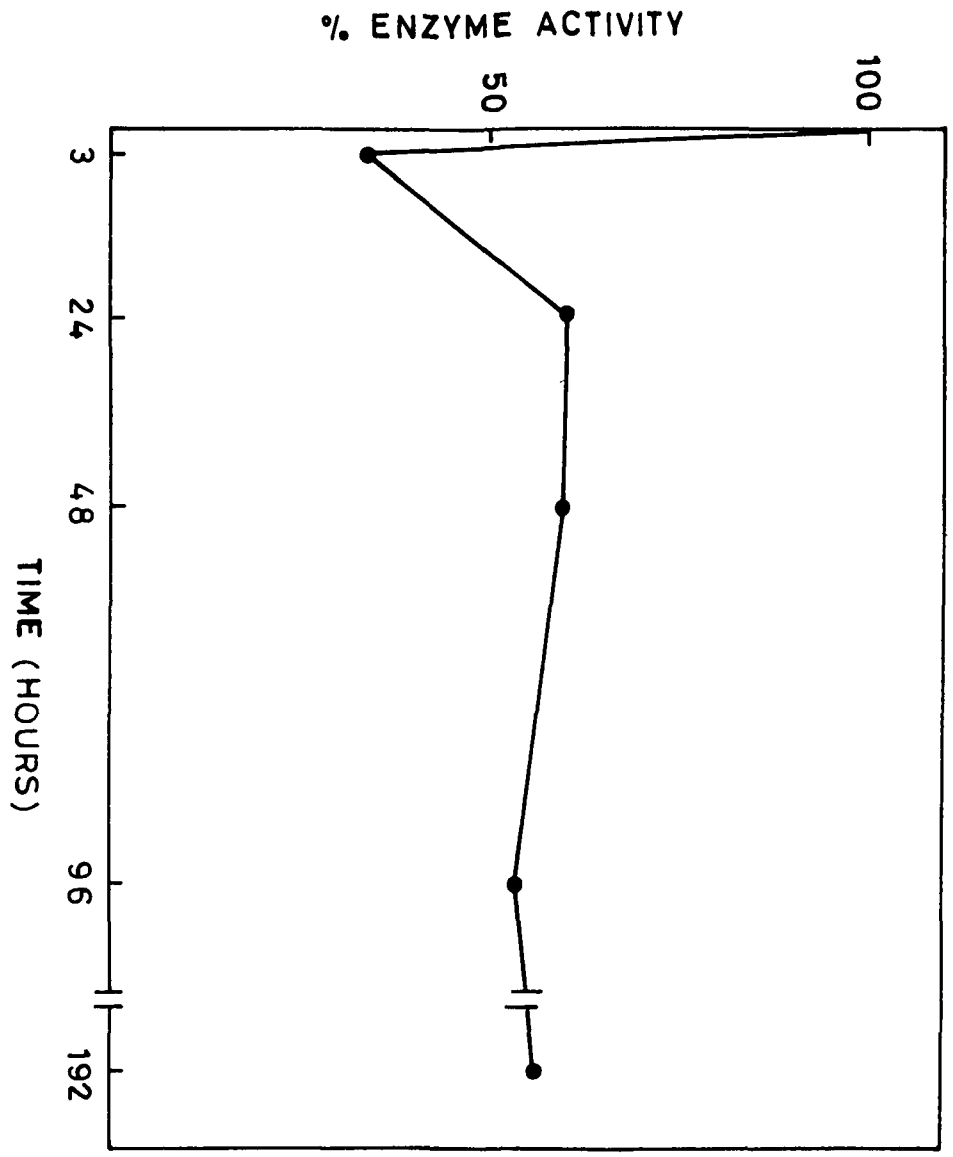
Mitochondria were incubated in Warburg flasks at 37°C. The incubation mixture in a total volume of 3.0 ml contained 100 µmoles sodium phosphate buffer, pH 7.4; succinate, 150 µmoles; cytochrome C(red), 4×10^{-5} µmoles; $CaCl_2$, 1.2 µmoles and $AlCl_3$, 1.2 µmoles. The reaction was started by tipping 0.4 ml Cyt. C from the side arm. The centre cup contained 0.2 ml of 2N NaOH. The enzyme activities were calculated as average $\mu l O_2/hr/mg$ of mitochondrial protein. The average enzyme activity in untreated (control) animals for different time points was 186 ± 5 $\mu l O_2/hr/mg$ of mitochondrial protein.

Time (Hrs.)	Enzyme Activity	Per cent Enzyme Activity
3	63 ± 2	34
24	111 ± 1	60
48	110 ± 4	59
96	98 ± 2	53
192	105 ± 4	56

$$\text{Percent enzyme activity} = \frac{\text{Enzyme activity in treated animals}}{\text{Enzyme activity in untreated animals}} \times 100$$

Figure 8. Effect of urethan on succinate-cytochrome C reductase of rat liver mitochondria

The data of Table VII has been plotted as a function of time


% ENZYME ACTIVITY
100
50
TIME (HOURS)
3
24
48
96
192

T A B L E - VIII

Effect of Cell Concentration on the Incorporation of Histidine into the Proteins of Hepatic Cells in Suspension

Hepatic cells, isolated from adult rat liver as described in methods, were incubated at 37°C in 6.0 ml of Ca^{++}-free Krbs Ringer Phosphate Buffer, pH 7.2 in experiments II, III and IV and in 3.0 ml of the buffer in experiment I, for 3.0 hours. The concentration of labelled Histidine was 0.0056 µmoles (0.44×10^6 d.p.m.)/ml in experiments I, II and IV and 0.0067 µmoles (0.52×10^6 d.p.m.)/ml in experiment III.

	Cell concentration X 10^6/ml	Specific Activity of Protein (CPM/mg dry wt.)
I.	0.34	113
	0.43	40
	0.86	38
	2.60	36
II.	0.041	161
	0.082	115
	0.246	82
	0.328	85
	0.82	51
	1.25	39
III.	0.125	237
	0.20	161
	0.25	146
	0.375	122
	0.75	93
	1.00	78
	1.125	67
	1.25	93
IV.	2.0	93
	3.0	79
	4.0	49
	5.0	51
	6.0	27

T A B L E - IX

Effect of Cell Concentration on the Transport and Incorporation of Histidine into the Proteins of Hepatic Cells in Suspension

Hepatic cells isolated from adult rat liver as described in methods were incubated at 37°C in 6.0 ml of Ca^{+} free Krebs Ringer Phosphate buffer pH 7.2 for 3 hrs. The concentration of labelled Histidine was 0.0056 µmoles (0.44×10^6 dpm)/ml. Free amino acid pool of hepatic cells was isolated and chromatographed on Whatman paper No.1. The radioactivity in the Histidine spot was determined after eluting the amino acid with 60% ethanol.

	Cell Conc. x 10^6/ml	Specific Activity of Protein, CPM/mg dry/wt.	Total Radioactivity in/amino acid pool (CPM/10^6 cells/ml)	Total Radio activity in Histidine(CPM)
I.	0.26	469	5115	4615
	0.53	306	2641	1886
	1.06	365	877	566
	1.59	254	713	390
	2.12	163	643	130
II.	0.2	58	675	595
	0.4	34	275	240
	0.8	23	150	121
	1.2	21	60	47
	1.6	16	108	85
III.	0.33	60	6000*	--
	1.00	26	1750*	--
	1.66	22	2000*	--
	2.00	24	1080*	--

* The free amino acids were estimated by ninhydrin method and the values expressed are CPM/mg of leucine.

T A B L E - X

Effect of Cell Concentration on the Transport and Incorporation of Methionine into the Proteins of Hepatic Cells in Suspension

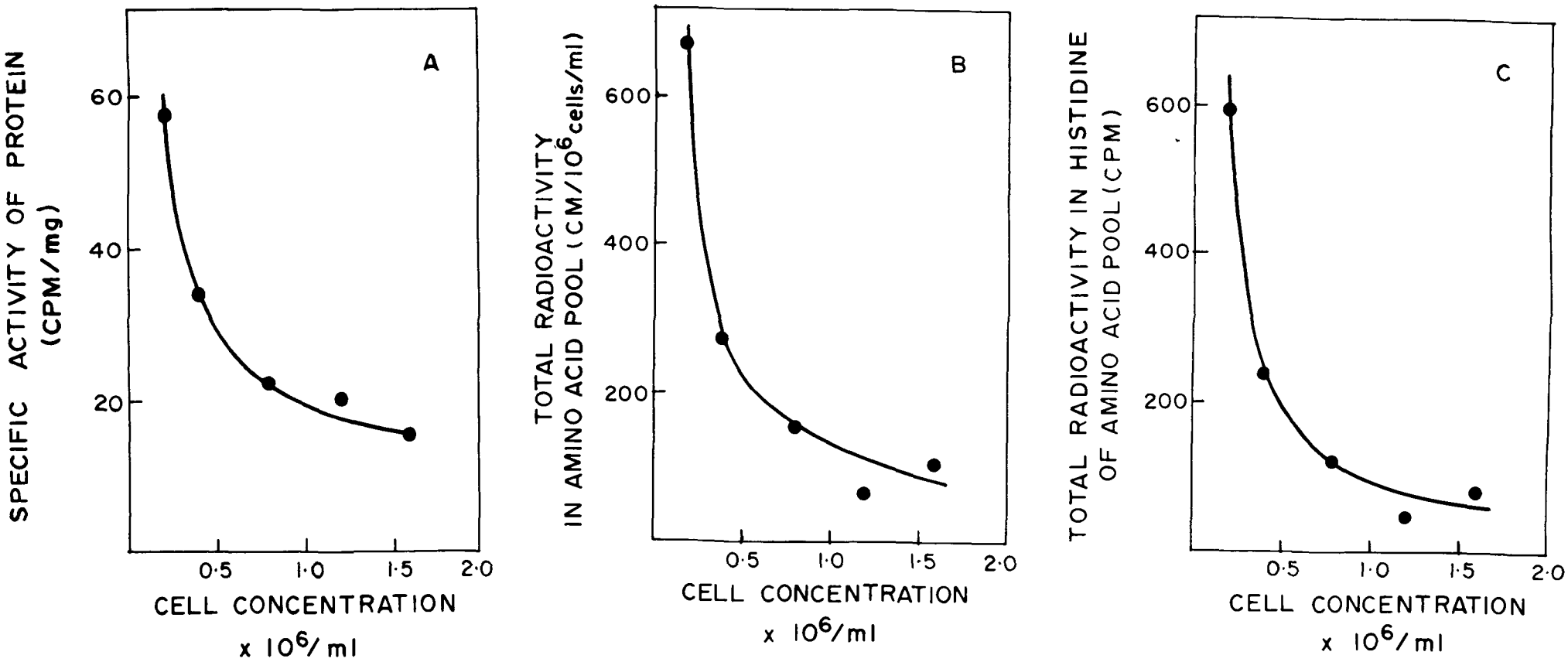
Hepatic cells isolated from adult rat liver as described in methods, were incubated at 37°C in 6.0 ml of Ca^{++}-free Krbs Ringer Phosphate buffer pH 7.2 for 3 hrs. The concentration of labelled Methionine was 0.00925 µmoles (0.55×10^6 dpm)/ml in experiment I and 0.0074 µmoles (0.44×10^6 dpm)/,l in experiment II. The free amino acids of the cells were isolated the radioactivity determined, as described in methods.

	Cells concentration x 10^6/ml	Specific Activity of Protein (CPM/mg dry wt.)	Total Radioactivity in free amino acid pool(CPM/10^6 cells/ml)
I.	0.29	78	690
	0.58	52	250
	1.16	46	262
	1.74	45	66
	2.32	38	32
II.	0.21	141	531
	0.43	74	94
	0.86	-	75
	0.29	48	-
	1.72	32	60

Figure 9. Effect of cell concentration on the transport and incorporation of l-Histidine-C^{14} into the proteins of hepatic cells in suspension

Experiment II of Table IX has been plotted as a function of cell concentration

A. Incorporation of l-Histidine-C^{14} into proteins
B. Total radioactivity in free amino acid pool
C. Total radioactivity in Histidine spot on paper chromatogram.


A
SPECIFIC ACTIVITY OF PROTEIN (CPM/mg)
60
40
20
0·5
1·0
1·5
2·0
CELL CONCENTRATION
x 10⁶/ml
B
TOTAL RADIOACTIVITY IN AMINO ACID POOL (CM/10⁶cells/ml)
600
400
200
0·5
1·0
1·5
2·0
CELL CONCENTRATION
x 10⁶/ml
C
TOTAL RADIOACTIVITY IN HISTIDINE OF AMINO ACID POOL (CPM)
600
400
200
0·5
1·0
1·5
2·0
CELL CONCENTRATION
x 10⁶/ml

b. Incorporation of L-Histidine-C^{14} into the Proteins of Hepatic Cells in Suspension and Perfused Liver Slices at different Cell Concentrations and Wet Weight respectively - The data of Table XI show that there is almost no effect of the wet weight of perfused liver slices on the incorporation of L-Histidine-C^{14} into the proteins. In the case of hepatic cells prepared from the same tissue, there was considerable effect of cell concentration. The maximum tissue concentration was 210 mg wet weight/6 ml (26.2 mg dry wt/ml) and maximum dry weight of hepatic cells used in these studies was 9.88 mg/ml.

c. Estimation of Total Free Amino Acid Pool in Hepatic Cells in Suspension Perfused and Uperfused Liver Slices - The total free amino acid pool has been estimated in hepatic cells in suspension, perfused and unperfused liver slices. The results (Table XII) indicate that the cells in suspension do not leak out any free amino acids during preparation and they are capable of retaining sufficiently high concentration of free amino acids.

d. Effect of Leakage of Amino acids from Cells on the Incorporation of Labelled Amino Acids into the Proteins of Hepatic Cells in Suspension

In order to check whether the leakage of free amino acids from the cells is responsible for the lower incorporation of radioactivity into proteins, samples of high and low concentration cells were incubated for 1½ hours. They were centrifuged and the incubation medium was interchanged. Cells

T A B L E - XI

Incorporation of Histidine into the Proteins of Hepatic Cells and Perfused Liver Slices at various Cell Concentration and Wet Weight respectively

Hepatic cells and liver slices taken from the same liver were prepared as described in methods and were incubated at 37°C in 6.0 ml of Ca^{++}-free Krebs Ringer Phosphate buffer pH 7.2 for 3 hrs.. The concentration of labelled Histidine was 0.0056 µmoles (0.44×10^6 dpm)/ml. Proteins were isolated as described in methods.

	Cell Concentration $x10^6$/ml	Wet weight of tissue in mgs.	Specific Activity of Protein (CPM/mg dry wt.)
I.	1.1	--	41
	3.3	--	23
	5.5	--	17
	6.6	--	17
II.	-	51	32
	-	100	14
	-	124	13
	-	171	14
III.	1.28	--	46
	3.84	--	19
	6.40	--	16
	7.68	--	20
IV.	-	91	17
	-	120	19
	-	170	15
	-	210	16

T A B L E - XII

Estimation of Total Free Amino Acid Pool from Hepatic Cells, Perfused and Unperfused Liver Slices

Hepatic cells and perfused and unperfused liver slices were prepared from adult rat liver and total free amino acids from these preparation were isolated as described in methods. Amino acids were estimated by Ninhydrin method taking leucine as standard.

Tissue	Total No.of Hepatic cells or wet weight of liver slices in mg.	Corresponding dry wt in mgs	Total free amino acids in µgm	µgms of amino acids per mg dry wt	Mean (µgm per dry wt)
Hepatic cells in suspension	$38x10^6$	31.1	152	4.88	5.05
	$76x10^6$	62.2	330	5.23	
Perfused liver slices	139	104.25	360	3.45	3.56
	61.5	46.12	170	3.72	
Unperfused liver slices	72.2	54.15	124	2.2	2.2
	242	181.5	400	2.21	

were then incubated in the presence of labelled phenylalanine. As shown in Table XIII, the results indicate that there was no effect of leakage of amino acids, breakage of cells or accumulation of toxic products if any on the cell concentration effect in the incorporation of amino acids into proteins of hepatic cells in suspension.

e. Effect of Cell Concentration on the Transport and Incorporation of 1-Histidine-C^{14} and other Labelled Amino Acids in Yoshida Sarcoma Cells - As shown in Tables XIV and XV and Figures 10 (A and B), the specific activity of protein and free amino acids was inversely proportional to the concentration of Yoshida sarcoma cells in the incubation medium. The range of cell concentration used in different experiments was between $1x10^6$ to $12x10^6$ cells/ml.

T A B L E - XIII

Effect of Leakage of Amino Acids from Cells on the Incorporation of Amino Acids into Proteins of Hepatic Cells in Suspension

$5x10^6$ cells and $25x10^6$ cells were incubated separately in 2.0 ml of Ca^{++}-free KRP buffer, pH 7.2 at 37°C. After 1½ hour incubation at 37°C, the samples were centrifuged. In case I the $5x10^6$ cells were resuspended in the incubation medium obtained from $25x10^6$ cells, In case II the reverse was done. In both cases .0029 µmoles phenylalanine-C^{14} ($1.11x10^6$ dpm)/ml were added and further incubated for 1½ hours at 37°C. The control samples containing same concentration of cells were incubated with same amount of phenylalanine-C^{14} for 3.0 hours at 37°C. The proteins were isolated by TCA method as described.

	Cell Concentration ($x10^6$)	Specific Activity of Protein (CPM/mg dry wt)
Control	5	517
	25	138
Case I	5	419
Case II	25	106

T A B L E - XIV

Effect of Cell Concentration on the Transport and Incorporation of a Mixture of Amino Acids into the Proteins of Yoshida Sarcoma (ascites) Cells

Yoshida Sarcoma (ascites) cells drawn from the peritoneal cavity of adult rats and purified as described in methods were incubated at 37°C in 10.0 ml of Ca^{++}-free Krebs Ringer Phosphate buffer pH 7.2 in experiment I and II and in 5.0 ml of the buffer in experiment III for 3 hrs. The concentration of Protein hydrolysate ($-C^{14}$) was 0.0078 µmoles (0.44×10^6 dpm)/ml and of mixture of four labelled amino acids (hist., ph. al., meth and thr.) was 0.748×10^6 dpm/ml.

	Cell Concentration $\times 10^6$/ml	Specific Activity of Protein (CPM/mg dry wt.)	Total Radioactivity in free amino acid pool (CPM/10^6 cells/ml)
I.	1.0	4922	5640
	2.0	2661	5390
	4.0	1921	3710
	6.0	1074	2816
	8.0	675	2903
	10.0	599	2407
II.	2.0	1455	--
	4.0	856	--
	6.0	693	--
	8.0	866	--
	10.0	858	--
	12.0	657	--
III.	1.6	2195	4440
	3.2	1492	3080
	4.8	1182	308
	6.5	790	440
	8.2	891	412

TABLE - XV

Effect of Cell Concentration on the Incorporation of Histidine into the Proteins of Yoshida Sarcoma (ascites) Cells

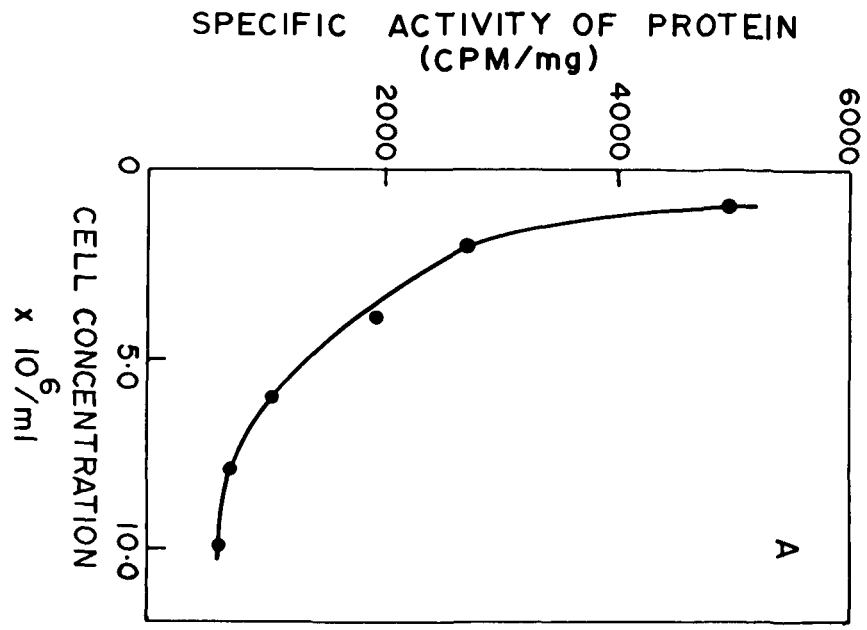
Yoshida Sarcoma (ascites) Cells drawn from the peritoneal cavity of rats and purified as described in methods, were incubated at 37°C in 10.0 ml of Ca^{++}-free Krebs Ringer Phosphate buffer, pH 7.2 for 3 hrs. The concentration of labelled Histidine was 0.00501 µmoles (0.396×10^6 dpm)/ml.

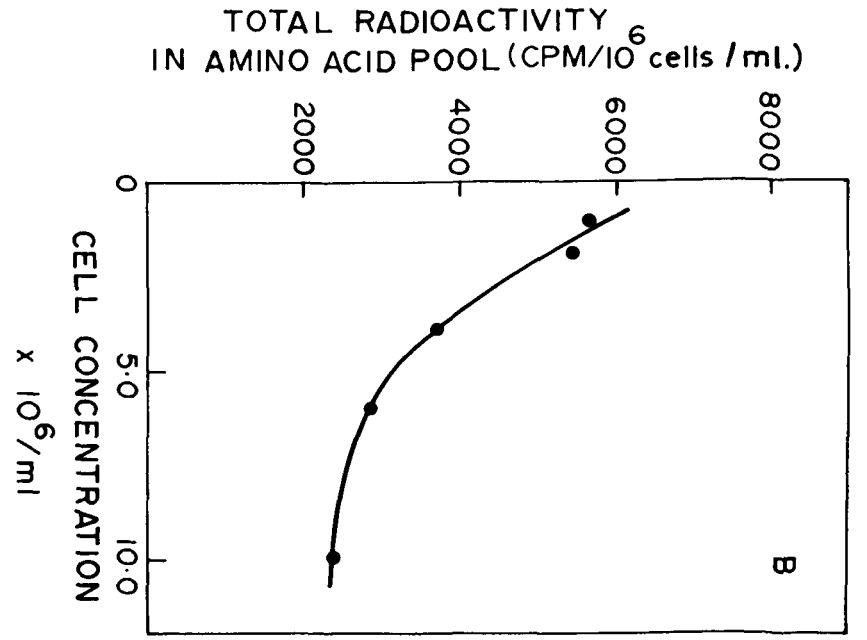
Expt. No.	Cell Concentration $\times 10^6$/ml	Specific Activity of Protein (CPM/mg dry wt.)
I.	0.7	237
	1.05	195
	1.4	55
	1.75	79
II.	2.34	56
	3.9	17
	7.8	10
	11.7	9
	15.6	8
III.	0.48	31
	0.96	20
	1.92	12
	3.84	8
	4.8	7
	5.76	7
	6.72	6
	7.68	6

Figure 10. Effect of cell concentration on the transport and incorporation of labelled amino acids into the proteins of Yoshida Sarcoma (ascites) cells

Experiment I of Table XIV has been plotted as a function of cell concentration

A. Incorporation of Protein-hydrolysate-C^{14} into proteins of Yoshida Sarcoma (ascites) cells
B. Total radioactivity in free amino acid pool.


SPECIFIC ACTIVITY OF PROTEIN
(CPM/mg)
2000
4000
6000
0
5·0
10·0
CELL CONCENTRATION
x 10⁶/ml
A


TOTAL RADIOACTIVITY
IN AMINO ACID POOL (CPM/10⁶ cells / ml.)
2000
4000
6000
8000
0
5·0
10·0
CELL CONCENTRATION
x 10⁶/ml
B

VII. DISCUSSION

1. INTERACTION OF URETHAN-C^{14} WITH TISSUE PROTEINS OF RATS AND MICE

Previous investigations on the mechanism of action of urethan using (1-C^{14})-ethyl carbamate and ethyl-(carbonyl-C^{14}) carbamate have indicated that over 90% of the C^{14} was removed from the animal body within 24 hours and only a small amount was retained (Skipper et al., 1951. Berenblum et al., 1958). Berenblum et al (1958) determined the subcellular distribution of Urethan-C^{14} and found that mitochondria concentrated more label than nuclei. They obtained similar results in in vitro studies. These authors, therefore, suggested that induction of pulmonary tumors is related to the uptake of urethan by lung mitochondria. However, these workers did not investigate the binding of urethan-C^{14} with protein or nucleic acids. Only recently Boyland and Williams(1969) have reported the interaction of urethan-C^{14} with nucleic acids and nuclear proteins. In the present investigation interaction of urethan-C^{14} with total tissue proteins of lung, liver and kidney tissues of rat and mice has been studied. Localization of urethan-C^{14} in nuclei and mitochondria of these tissues has also been investigated.

It is evident from the data presented in Tables I and II

that urethan-(Carbonyl-C^{14}) when administered intraperitoneally interacts with cellular proteins of lung, liver and kidney tissues of rats and mice. The time course study (Table II) of urethan-C^{14} interaction with total cellular proteins clearly indicates that the binding of urethan or its metabolite is time dependent. The maximum binding with lung proteins was observed 24 hours after the administration of urethan, whereas in the case of liver and kidney tissues maximum protein bound radioactivity was found at 48 hours and 12 hours respectively. The binding of urethan with proteins gradually declined after reaching the maximum and no radioactivity was found in protein samples at 192 hours, after the urethan-C^{1} administration (Figure 1). Similar time dependent pattern has also been observed in the binding of dimethylaminoazobenzene with proteins (Roberts and Warwick, 1966; 1967).

As shown in Fig. 1 the extent of binding of urethan was maximum with lung proteins whereas the binding of urethan with liver and kidney proteins was almost of the same order. The results are in agreement with the observations that lung is most susceptible tissue to urethan carcinogenesis (Mirvish, 1968). Similar correlations between the extent of binding of dimethylaminoazobenzene and 2-Acetylaminofluorine with proteins and the tissue susceptibility to Carcinogenesis have been obtained

earlier (Miller and Miller, 1952, 1953, 1966).

As regards the mode of binding of urethan or its metabolite with tissue proteins, the decrease in the specific activity of protein (CPM/mg) samples on heating (Tables I and II) strongly suggested a non-covalent interaction. The loss of radioactivity also rules out the possibility of its incorporation into proteins. From structural considerations both carbonyl and amino groups of urethan are capable of taking part in the fromation of hydrogen bonds with free amino and carbonyl groups in proteins. It is, therefore, possible that urethan-protein interaction may cause conformational changes in the tissue proteins.

A high degree of urethan-C^{14} binding with cellular proteins of lung, liver and kidney tissues of younger mice (Table II) than older mice (Table III) may possibly be due to the difference in the rate of catabolism of urethan in the two groups of animals. It is known that as compared to young animals urethan is removed much faster in older ones (Nery, 1968). This view is supported by the observaiion of high incidence of tumors development in younger animals (Marvish, 1963). It is, therefore, suggestive that the length of time, urethan remains in the body, is a critical factor in the initiation of carcinogenesis.

The study on inracellular distribution of urethan-C^{14} showed

much more concentration of urethan in mitochondria than nuceli (Table IV). In the case of liver the ratio of its distribution into nuclei and mitochondria was found to be 1:6. These observations are in agreement with the finding of Beremblum et al (1958).

From these studies it can be concluded that: (a) carcinogenic potency and tissue specificity of urethan is directly related to the extent of its interaction with tissue proteins in mice, (b) urethan-protein interaction is temporary and heat labile and (c)urethan is concentrated much more in mitochondria than in nuclei, suggesting an important role of micochondria in carcinogenesis.

2. EFFECT OF THIOACETAMIDE AND URETHAN ON SWELLING AND CONTRACTION CYCLE AND SUCCINATE-CYTOCHROME C REDUCTASE OF RAT LIVER MITOCHONDRIA -

As discussed above urethan has been shown to go preferentially to mitochondria when given in carcinogenic dose to mice. This suggest a direct interaction of chemical carcinggens with mitochondria and it is possible that such an interaction may cause various changes in the biochemical activity of these organelles. Swelling and contraction cycle of mitochondria is known to reflect the structural state of mitochondrial membrances and its transport properties. Therefore, the effects of thioacetamide and urethan on swelling and contraction cycle were studied.

The results obtained indicate that thioacetamide and urethan, which are potent chemical carcinogens for rats are able to affect, both *in vitro* and *in vivo*, the swelling and contraction properties of rat liver mitochondria. The observations show that these compounds are good swelling agents of rat liver mitochondria. Thioacetamide induced mitochondrial swelling at 20°C is dependent on the concentrations of the inducer as well as mitochondria in the medium (Fig. 3, A & B). This thioacetamide induced swelling is inhibited by 2,-4-dinitrophenol (Fig.4). Therefore the process appears to be linked to oxidative phosphorylation. Under similar conditions urethan has been found to induce mitochondrial swelling which is dependent on its concentration (Fig.5). However, it was not as prominent as with thioacetamide.

Thioacetamide and urethan have also been found to stimulate the spontaneous swelling on mitochondria at 37°C Fig. 2A and B. However, the contraction of mitochondria was inhibited in these cases (Table V). The swelling of mitochondria could occur, in addition to other factors, due to an interaction of these carcinogens with mitochondrial membrance proteins resulting in an altered conformation which favours the intrusion of water. Inhibition of the reversal of mitochondrial swelling observed in these cases could possibly occur as a result of (a) an irreversible

interaction of these compounds with structure controlling groups in the mitochondrial membrane which renders it incapable of acquiring the original state during contraction process, and (b) the contraction factor (Protein in nature) is either lost during the incubation of mitochondria with thioacetamide or urethan, or it is affected by interacting with these compounds and becomes partially inactive.

The in vivo study shows that mitochondria obtained from rats which were given carcinogenic doses of thioacetamide and urethan 24 hours prior to killing, failed to response at 20°C to inorganic phosphate induced swelling properly. This observation could be explained on the basis of in vitro experiments which show that thioacetamide and urethan are capable of inducing mitochondrial swelling. It is, therefore, possible that on treatment of rats with these compounds the mitochondria are initially swellen and thus could not undergo swelling to the same extent as mitochondria isolated from untreated animals (Fig.6). Similar effects have been found in the case of aminoazo dyes (Clerici and Cudkowicz, 1956; Emmelot and Bos 1957). Tumor mitochondria are shown to exist in swollen state and therefore could not response to large amplitude swelling induced by phosphate and other agents to the same

extent as liver mitochondria (Feo, 1967).

It can thus be concluded from these considerations that thioacetamide and urethan which are potent carcinogens affect the swelling and contraction properties of rat liver mitochondria. As this might occur as a result of alterations in the structure of mitochondrial membrane proteins, it is possible that these compounds may influence the permeability and transport properties of mitochondria, which may eventually lead to the overall changes observed in the mitochondria from tumor cells. This view is supported by the reports that changes in the swelling properties of mitochondria are directly related to the onset of carcinogenesis during 3-methyl-dimethyl-aminoazo benzene feeding (Arcose et al.,1958, 1960, 1961, 1969).

Since thioacetamide and urethan affect mitochondrial swelling and contraction, the in vivo effect of these carcinogens on succinate-cyt C reductase, a key enzyme of mitochondria, was also studies. As shown in Figure 7, there is a sharp decrease in the enzyme activity at 3 hrs. after the administration of thioacetamide. The decrease afterwards is gradual and the lowest enzyme activity observed is 28 percent of the initial activity at 48 hours (Table VI). In the case of the urethan the maximum inhibition was found to be at 3 hours when it is known to be present in the

body to a considerable extent. The enzyme activity increased slightly after 3 hours and became steady at about 60 percent of the original activity after 24 hours (Figure 8). In case of thioacetamide the inhibition was more prominent. However, the inhibition of enzyme activity was found to be 34% and 44% after 192 hours of the administration of thioacetamide and urethan respectively. In a separate experiment 45 percent inhibition was observed even 10 days after the urethan treatment. These observations are of much interest in view of the fact that both these compounds are rapidly catabolized in rats and about 90-95% of the dose is removed from the animal body within 24 hrs. (Bryan et al., 1949; Rees et al., 1966). Therefore, it rules out the possibility that the presence of these compounds per se is affecting the enzyme activities. There can thus be two possible explanations for the observed decrease in enzyme activities; (a) that thioacetamide and urethan or their metabolites combination with the enzyme brings about conformational changes in the protein which render the enzyme partially inactive, and (b) that the modification of enzyme activity may be due to the structural changes in the mitochondria. This possibility is based on the fact that changes in the swelling and contraction properties of mitochondria are known to affect the enty and

retention of certain important constituents of mitochondria, such as NAD (Gause, 1967). Similar situation may exist in tumor mitochondria where decrease in mitochondrial swelling is accompanied by enzymatic changes in mitochondria.

It can thus be concluded from these studies that thioacetamide and urethan when administered in rats effectively alter the respiration and the activity of succinate-Cytochrome C reductase in mitochondria. These observations together with the swelling and contraction studies highlight the importance of early biochemical changes in mitochondria in thioacetamide and urethan carcinogenesis.

3. CELL CONCENTRATION EFFECT ON THE TRANSPORT AND INCORPORATION OF LABELLED AMINO ACIDS IN HEPATIC CELLS IN SUSPENSION AND YOSHIDA SARCOMA CELLS

The rate of incorppration of labelled amino acids in bull spermatozoa and in rabbit reticulocytes has been found to be inversely proportional to the concentration of these cells in the incubation medium (Bhargava et al., 1959). Similar effect was observed earlier for respiration in spermatozoa (Bishop and Salisbury, 1955). Hlade et al (1966) found a similar concentration effect on the incorporation of P^{32} into hamster cells cultivated in suspension and mouse ascites tumor cells. It seems possible that the concentration effect may be a general phenomenon in

mammalian cells, due to the intercellular contacts. Therefore, the effect of cell concentration on the transport and incorporation of labelled amino acids in rat liver parenchymal cells and Yoshida Sarcoma (ascites) cells was studied.

The results reported here show that the uptake and incorporation of labelled amino acids into the proteins of hepatic cells in suspension in dependent on the concentration of hepatic cells in the medium. This phenomenon has been called as the cell concentration effect (Bhargava and Bhargava 1962). The possibility that the observed cell concentration effect is, due to (a) the limiting factors such as oxygen and amino acids (b) production of toxic substances and (c) a decrease in the specific activity of labelled amino acids in the medium due to leakage of amino acids from the cells or due to lysis of some cells during incubation, is ruled out on the basis of the following observations.

(i) The total amount of labelled amino acids transported and incorporated into the proteins of cells, at any cell concentration used, represented a negligible proportion of the amount present initially or at the end of the incubation, in the medium as checked experimentally (Bhargava, 1970).

(ii) No such concentration effect was found in the case of liver slices (Table XI).

(iii) No concentration effect was found in the respiration of hepatic cells (Reddy, 1969)

(iv) The experiments described in Table XIII completely rule out the possibility of toxic substances, leakage of amino acids or lysis of cells, making any contribution in the phenomenon of cell concentration effect.

The fact that the rate of transport of labelled amino acids into the hepatic cells was also inversely proportioned to the cell concentration in the medium (Tables VIII, IX, X; Figure 9), and other available evidences clearly indicate that the cell concentration effect is not due to intracellular or extracellular factors (Bhargava and Bhargava, 1962). Therefore the observed phenomenon may be due to intercellular factors. Since transport of nutrients into the cells is known to be a function of membrance activity, enhanced contact between cells is likely to inhibit the transport of labelled amino acids either by causing overall reduction in functional biochemical activity of the membrance or a reduction in the cell surface area to which the amino acids have an access. Thus the lowering of transport and incorporation of amino

acids into the hepatic cells is most likely due to the frequency of direct contacts between cells.

Similar observations are made with Yoshida Sarcoma (ascites) cells, which are single tumor cells grown in the peritoneal cavity of rats (Tables XIV, XV Fig. 10A and B). However, the cell concentration used here was much higher than in the case of hepatic cells in suspension.

The studies on the estimation of total free amino acids in hepatic cells in suspension, perfused and unperfused rat liver slices (Table XII) indicate that there is no leakage of amino acids from the hepatic cells during preparation. It is also suggestive from these studies that the hepatic cells are capable of retaining sufficiently high concentration of amino acids.

It can thus be concluded that increased frequency of cell contacts cause a reduction in the rate of transport of amino acids in hepatic cells in suspension and Yoshida Sarcoma cells and that this is a likely explanation for the observed decrease in the incorporation of labelled amino acids into the proteins when the concentration of cells was increased in the medium. Thus, contacts between cells could not be a factor regulating the metabolic activity in mammalian cells (both normal and concerous).

VIII. SUMMARY

It has been demonstrated that urethan which is structurally one of the most simple chemical carcinogen interacts with total tissue proteins of lung, liver and kidney tissues of rats and mice. The maximum binding with lung protein was observed 24 hours after the administration of urethan, whereas in the case of liver and kidney tissues maximum protein bound radioactivity was found, at 48 hours and 12 hours respectively. The binding of urethan with proteins gradually declined after reaching the maximum and no radioactivity was found in protein samples at 192 hours, after the urethan administration. As compared to liver and kidney proteins, the extent of urethan binding was more with lung proteins which is in agreement with earlier observations that lung is the most susceptible tissue to urethan carcinogenesis. The data obtained strongly suggest a non-covalent interaction of urethan with tissue proteins. The age of animals has been found to be determining factor in urethan protein interaction. As compared to older animals (12-16 weeks) the interaction was more in younger animals (3-4 weeks). Since urethan is known to be catabolized much faster in older animals, it is suggested that the length of time, urethan remains in the animal body may be a critical factor in the initiation of carcinogenesis. The

study on intracellular distribution of urethan-C^{14} showed much more concentration of the carcinogen in mitochondria than nuceli.

The preferential concentration of urethan in mitochondria is suggestive of an important role of mitochondria in carcinogenesis. Mitochondrial swelling and contraction is known to reflect the structural state of mitochondrial membrane and its transport properties. Therefore the effect of thioacetamide and urethan on swelling and contraction cycle of mitochondria was studied. It has been observed that both thioacetamide and urethan induce mitochondrial swelling but inhibit its contraction. Since 2,4-dinitrophenol inhibits thioacetamide induced swelling the process may be energy linked. As compared to control animals, inorganic phosphate i induced swelling of rat liver mitochondria from thioacetamide and urethan treated animals was inhibited. These results are in agreement with earlier reports where swelling of mitochondria from 3-methyl-dimethylaminoazobenzene treated rats was inhibited.

Since thioacetamide and urethan affected <u>in vivo</u> swelling of mitochondria, the <u>in vivo</u> effect of these carcinogens on succinate-cytochrome C reductase, a key enzyme of mitochondria was also studied. The results show that single intraperitoneal injections of carcinogenic doses of these compounds bring about a significant decrease in succinate-cytochrome C reductase

of rat liver mitochondria.

In order to understand the role of cell contact and intercellular material in determining the biochemical properties in mammalian cells, the effect of cell concentration on the transport and incorporation of labelled amino acids into the proteins of hepatic cells in suspension and Yoshida Sarcoma (ascites) cells was studied. It has been found that the rate of transport and incorporation of labelled amino acids into the proteins decreases when the concentration of hepatic cells in the medium is increased. However, there was no evidence of leakage of amino acids or accumulation of toxic products in the medium. Similar results were obtained with Yoshida Sarcoma (ascites) cells. These observations suggest that increased frequency of cellular contact is responsible for the reduction in the rate of transport of amino acids in hepatic cells in suspension and Yoshida Sarcoma (ascites) cells. Therefore cell contact could be a factor regulating the metabolic activities in mammalian cells.

IX. REFERENCE

The references marked with an asterisk (*) have not been seen in the original.

Abell, C.W. and Heidelberger, C. (1962). Cancer Res. 22, 931

Aisenberg, A.C. (1961). The Glycolysis and Respiration of tumors, Academic Press, New York.

Allard, C.R., Mathieu, G., De Lumurande and Cantero, A.(1952). Cancer Res. 12, 407

Anderson, N.G.(1953). Science. 117, 627

Arcos, J.C. and Arcos, M. (1958) Biochem. biophys. Acta. 28, 9

Arcos, J.C., Griffith, G.W. and Cuningham, R.W.(1960). J.Biophys. Biochem. Cytol 7 , 49

Arcos, J.C., Griffith, G.W. and Cuningham, R.W.(1961). J.Biophys. Biochem. Cytol 10, 23

Arcos, J.C., Mathison, J.B., Tison, M.J. and Mouledous, A.M. (1969) Cancer Res. 29, 1288

Attardi, G. and Attardi, B. (1968). Proc. nat. Acad. Sci., Wash., 61, 261

*Azzone, G.F. and Azzi, A. (1965). Proc. Symp. on Regul. Met. Processes in Mitochondria., 1965, pp. 332

Bahr, G.F. and Zeitler, E. (1962). J.Cell Biol. 15, 489

Bates, R.R., Wortham, J.S., Counts, W.B., Dingman, C.W. and Gelboin, H.V.(1968) Cancer Res. 28, 27

Berenblum, I., Haran-Ghera, N., Winnick, R and Winnick, T (1958) Cancer Res. 18, 181

Berwald, Y. and Sachs, L. (1963). Nature, 200, 1182

Beyer, R.E., Ernester, L., Low, H. and Beyer, T. (1955) Expt. Cell Res. 8, 586

Bhargara, K. and Bhargava, P.M. (1962). Life Sci. 477

Bhargava, P.M. (1968) Sci. Cult, Calcutta, 34 (Suppliment), 105

Bhargava, P.M. (1970). Ciba Found. Symp. on Control Processes in Multicellular Organisms, J.& A. Churcill Ltd., London, p. 158

Bhargava, P.M., Bishop, M.W.H. and Work, T.S. (1959) Biochem. J. 73, 267

Bishop, M.W.H. and Salisbury, G.W. (1955 a). Am.J. Physiol. 180, 107

Bishop, M.W.H. and Salisbury, G.W. (1955b). Am.J. Physiol. 181, 114

Blade, E., Harel, L. and Hanania, N. (1966). Exp. Cell Res. 41, 473.

Boyland, E. and Nery, R. (1965). Biochem. J. 94, 198

Boyland, E. and Rhoden, E.(1949). Biochem. J. 44, 528

Boyland, E. and Williams, K. (1969). Biochem. J. 111, 121.

* Brill, E. and Rodbnski, J.L. (1965) Biochem. Pharmacol. 14, 743

Bryan, C.E., Skipper, H.E., and White, L. (1949) J. Biol. Chem. 177, 941

Buch, C.A. and Nass, M.M.K. (1968). Proc. nat. Acad. Sci., Wash.,60, 1045

Buch, C.A. and Nass, M.M.K. (1969). Proc. nat. Acad. Sci., Wash.,62, 506

Burk, D., Woods, M. and Hunter, J.(1965) Proc. Am. Assoc. Cancer Res.6, 9

Burk, D., Wood, M. and Hunter, J.(1967). J.Nat.Cancer Inst. 38, 837

Chance, B and Packer. L. (1958). Biochem. J. 68, 295

Calvin, M., Heidelberger, C., Reid, J.C., Tolbert, B.M. and Yankwick, P.F.(1949). Isotopic Carbon, P.288, J.Wiley & Sons Inch., New York

Clayton, D.A. and Vinograd, J. (1969) Proc. nat. Acad. Sci., Wash 62, 1077

Ceiand, E. (1952). Nature, Lond., 170, 497

Clerici, E and Cudkowicz, G. (1956). J. Nat. Cancer Inst. 16, 1459

Comorosan, S., Gasper, A and Sandru, D.(1968). Biochem. Biophys Acta. 166, 394

Constantin. T (1964). Comp. Rend. Soc. Biol. 158, 2263

Dawid, I.B. and Wolstenlolme, D.R. (1967). J.Mol. Biol. 28, 233

Dawson, R.M.C., Elliot, D.N., Elliot, W.C. and Jones, K.M. (1959) Data for Biochemical Research, p. 209. Oxford University Press,London.

De Bann, J.R., Miller, E.C. and Miller, J.A. (1967). Proc. A.M. Assoc. Cancer Res. 8, 12

Dickens, F. and Salmony, D. (1956). Biochem. J. 64, 645

Dubois, K.P. and Potter, V.R. (1943). Cancer Res. 3, 353

Dulbecco, R and Vogt, M (1954). J.Exp. Med. 99, 167

Dyer, H.M. and Morris, H.P. (1956). J. Nat. Cancer. Inst. 17, 677

Eisenberg, S., Be,-or, S. and Doljanski, F. (1962). Exp. Cell Res. 26,451

Emmelot, P. and Bos, C.J. (1957). Exp. Cell Res. 12, 191

* Ephrussi, B., Hottinguer, H and Chimenes, A.M. (1949) Ann. Inst. Pasteur, 76, 351

Farber, E. (1963). Adv. in Cancer Res. 7, 303

Ferber, E (1968). Cancer Res. 28, 1859

Feo, F. (1967). Life Sci. 6, 2417.

Fiala, S., and Fiala, A (1966) Proc. Am. Assoc. Cancer Res. 7, 20

Fiala S and Fiala, A. (1967) Life Sci. 6, 2417

Freed, J.J. and Sorof, S. (1966). Biochem. biophys. Res. Commun. 22, 1

Gause, G.F. (1967), Adv. in Applied Microbiol, Vol. 9, Acad. Press, New York P. 69

Gelboin, H.V. (1967) Adv. in Cancer Res. 10, 1

Goldblatt, M.W., Henson, A.F. and Somerville, A.R. (1960). Biochem. J. 77, 511

Graffi, A., Butschak, G and Schneider, E.S. (1965) Bioch. biophys. Res. Comm., 21, 418

Granick, S and Gibor, A. (1967). Proc. of Symp. on Nucleic Acid Res. and Mol. Biol., Vol. 6, Edrs. J.N. Davidson and W.E. cohen, Acad. Press., New York, p. 143

Grant, H.C., and Rees, K.R. (1958). Proc. Royal. Soc. B. 148, 117

Gupta, D.N. (1956). J.Path. Bact. 72, 181

Harrison, M.F. (1953 a). Proc. Royal Soc. B 141, 203

Harrison, M.F. (1953 b). Nature. Lond., 171, 611

Heidelberger, C. and Ipe, P.T. (1967) Science, 155, 214

Hennings, H., Colburn, H.H. and Boutwell, R.K. (1968). Cancer Res. 28, 543

Henson, A.F., Somerville, A.R., Farquharson, M.E. and Goldblatt, M.E. (1954) Biochem. J. 58, 383

Higgins, J.A. (1967). Int. Lipid Res., 8, 636

Higgins, J.A. and Green, C (1966). Biochem J. 99, 631

Hoffsten, P.E., Hunter, F.E. Jr., Gebicki, J.M. and Weingtein, J. (1962) Biochem Biophys. Res. Commun. 7, 276

Hudson, B and Vinograd. J. (1967) Nature, Lond. 216, 647, 652

Hunter, F.E. Jr., Davis, J. and Carlat, L. (1956). Biochem. biophys Acta. 20, 237

Hunter, F.E. Jr., and Ford, L (1955). J. Biol. Chem. 216. 357

Hunter, F.E. Levy, J.F., Findk, J., Schutz, B., Guerra, F and Hurwitz, A. (1959). J. Biol. Chem. 234, 2176

Hunter, F.E. Jr., Gebicki, J.M., Hoffsten, P.E., Weinstein, J.and Scott, A. (1963). J./Biol Chem.238, 828.

Ipe, P.T. and Bhargava, P.M. (1965). Bioch. J. 94, 284

Ipe, P.T., Bhargava, P.M. and Taskar, A.D. (1965). Exp. Cell Res. 40, 233

Jacob, S.T. and Bhargava, P.M. (1962). Exp. Cell Res. 27, 453

Jacob, S.T. and Bhargava, P.M. (1965). Biochem. J. 95, 568

Kadenbach, B. (1967). Biochem. Biophys. Acta. 134, 430; 138, 651

Kalf, G.F. (1964). Biochemistry. 3, 1702

Kalf, G.F. and Chik, J.J. (1968). J.Cell Biol. 39, 69 A

Karoon, A.M., Borst, P., Vanbruggen, E.F.J. and Ruttenberg, G.J.C. (1966) Proc. nat. Acad. Sci., Wash. 56, 1836

Kind, C.M. and Philips, B. (1968). Science. 159, 1351

Kovachevich, R. (1964). Biochem. Biophys. Res. Commun. 14, 48

Lehninger, A.L. (1959). J.Biol. Chem. 234, 2465

Lehninger, A.L. (1961). Proc. nat. Acad. Sci., Wash., 47, 1929

Lehninger, A.L. (1962). Physiol. Rev. 42, 467

Lehninger, A.L. (1964). The Mitochondrian', Benjamin.

Lehninger, A.L. and Remmert, L.F. (1959). J.Biol. Chem. 234, 2459

Lehninger, A.L. and Shneider, M (1958). Z.Physiol. Chem. 313, 138

Lowry, O.H., Rosenbrought, N.J., Farr, A.L. and Randell, R.J. (1951) J.Biol. Chem. 193, 265

Marroquin, F. and Farber, E. (1965). Cancer Res. 25, 1262

Matile, Ph., and Bahr, G.F. (1968). Exp. Cell Res. 52, 301

Mehrotra, B.D. and Mahler, H.R. (1968). Arch. Biochem. Biophys. 128, 685

Meyer, R.R. and Simpson, M.V. (1968). Proc. nat. Acad. Sci., Wash., 61, 130

Mifuchi, I., Morita, T., Yanagihara, Y., Hosoi, M. and Nishida, M. (1963) Japan J. Microbiol. 7, 69

Miller, E.C. (1951). Cancer Res. 11, 100

Miller, E.C. and Miller, J.A. (1947). Cancer Res. 7, 468

Miller, E.C. and Miller, J.A. (1952) Cancer Res. 12, 547

Miller, E.C. and Miller, J.A. (1953). Adv. in Cancer. Res. 1, 339

Miller, E.C. and Miller, J.A. (1955). J.Nat. Cancer Inst. 15, 1571

Miller, E.C. and Miller, J.A. (1966). Phamacol. Rev. 18, 805

Miller, L.A. and Goldfeder, A. (1965). Proc. Soc. Exp. Biol. Med., 119, 759

Mirvish, S.S. (1966). Biochem. Biophys. Acta. 117,1

Mirvish, S.S. (1968). Adv. in Cancer Res. 11,1

Modie, M.M., Reid, C and Wallick, C.A. (1954). Cancer Res. 14, 367

Moor, S and Stein, W.H. (1948). J. Biol. Chem. 176, 367

Morita, T. and Mufuchi, I. (1970). Biochem. Biophys. Res. Commun. 38, 191

Mounoulou, J.C., Jacob, H and Slonimski, P.P. (1966). Biochem. Biophys Res. Commun. 24, 218

* Moustacchi, E and Marcovich, H. (1963). Compt. Rend. 256, 5646

Mulolo, V and Abrignani (1957). Brit. J Cancer. 11, 590

Nakajima, A and Dander, P. (1964). Exp. Cell Res. 35, 434

Nass, M.M.K., Nass, S. and Afzelius, B.A. (1965). Exp. Cell Res. 37, 51

Natori, Y., Trowbridge, H.C. Toreson, W.E. and Tarner, H. (1961) J.Biol. Chem. 236, 2821

Neifakh, S.A., Avramov, J.A., Gaitskhoki, V.S., Kazakova, T.B., Monakhov, N.K., Repin, V.S., Turovski, V.S, and Vassiletz. I.M. (1965). Biochem. Biophys. Acta. 100, 329

Nery, R. (1968). Biochem. J. 106, 1

Neubert, D. and Lehninger, A.L. (1962). J. Biol. Chem. 237, 952

* Neubert, D., Helge, H. and Bass, R. (1965). Arch. Exp. Path. Pharmac. 252, 258

O'Brien, T.W. and Kalf, G.F. (1967). J. Biol. Chem. 242, 2172, 2180

Packer, L. (1959). Ann. N.Y. Acad. Sci. 72, 518

Price, C.A., Fonnesu, A. and Davies, R.E. (1956). Biochem. J. 64, 754

* Raaflaub, J. (1953). Helv. Physiol. Pharmacol. Acta. 11, 142

Radloff, R., Bauer, W. and Vinograd. J. (1967). Proc. nat. Acad. Sci., Wash., 57, 1514

Reddy, R.R. (1969). Ph. D. Thesis, Aligarh Muslim University, Aligarh

Rees, K.R., Rowland, G.F. and Varcoe, K.F. (1966). Int. J.Cancer. 1, 197

Reid, E. (1962) Cncer Res. 22, 398

Reich, E and Luck, D.J.L. (1966). Proc. nat. Acad. Sci., Wash., 55, 1600

Riley, M.V. and Lehninger, A.L. (1964). J. Biol. Chem. 239, 2083

Roberts, J.J. and Warwick, G.P. (1966). Int. J. Cancer, 1, 179

Roberts, J.J. and Warwick, G.P. (1967). Nature, Lond., 213, 1206

Sandstrom, B. (1965). Exp. Cell Res. 37, 552

Sapre, N.N., Ghotoskar, S.V. and Bhide, S.V. (1969). Ind. J.Exp. Biol. 7, 4

* Schatz, G., Tuppy, H. and Klima, J. (1963). Z.Naturforch. 18b, 145

Scheider, W.C. (1946). Cancer Res. 6, 695

Schneider, W.C. and Potter, V.R. (1943 a). J.Biol. Chem. 149, 217

Schneider, W.C. and Potter, V.R. (1943 b). Cancer Res. 3, 353

Scott, A. and Hunter, F.E. Jr. (1966). J.Biol. Chem. 241, 1060

Scribner, J.D., Millerk J.A. and Miller, E.C. (1965). Biochem. biophys. Res. Commun. 20,560

Sen, K.C. (1931). Biochem. J. 25, 849

Shanmugam, G and Bhargava, P.M. (1966). Biochem. J. 99, 297

Shanmugam, G. and Bhargava, P.M. (1969). Ind. J. Biochem. 6, 64

Skipper, H.E., Bennet, L.L., Bray, C.E., White, L., Newton. M.A. and Simpson, L. (1951). Cancer Res. 11, 46

Slonimski, P.P. and Hirch, H.M. (1952). Compt. Rend. 235,741

Smith, D., Tauro, P., Schweizer, E and Halvorson, H.O. (1968). Proc. nat. Acad. Sci. 60, 936

Somerville, A.R., Henson, A.F., Cooke, M.E., Farquharson, M.E. and Goldblatt, M.W. (1956). Biochem. J. 63, 290

Sorof, S. and Cohen, P.P. (1951). Cancer Res. 11, 376

Sorof, S., Cohen, P.P., Miller, E.C. and Miller, J.A. (1951). Cancer Res. 11, 383

Sorof, S., Young, E.M. and Fetterman, P.L. (1960). Exp. Cell Res. 86, 253

Sorof, S, Young, E.M. and Fetterman, P.L. (1962). Proc. Am. Assoc. Cancer Res. 3, 362

Sorof, S., Young, E.M. and Ott, M.G. (1958). Cancer Res. 18, 33

Sorof, S., Young, E.M., McCue, M.M. and Fetterman, P.L. (1963) Cancer Res. 23,685

Sorof, S. Young, E.M., Mcbridge, R.Z. and Coffey, C.B. (1965) Fed. Proc. 24, 683

Stagman, W.J., Cooper C.S. and Avers, C.J. (1970) Biochem. biophys. Res. Commun. 39, 69

Stekol, J.A. (1963). Ad. in Enzymol. 25, 369

Suyama, Y. (1967). Biochemistry, 6, 2829

Svoboda, D. and Higginson, J. (1968). Cancer Res. 28, 1703

Tapley, D.F. (1956). J. Biol. Chem. 222, 325

Truman, D.E.S. (1963) Exp. Cell Res. 31, 313

* Warburg, O. (1923). Biochem. Z. 142, 317

Webb, J.A. and Green, C (1966), Biochem. J. 91, 24P

Weisburger, J.H., Crantham, P.H., Morris, H.P. and Weisburger, E.K. (1961). J.Nat. Cancer Inst. 27, 153

Whitcutt, J.M., Sutton, D.A. and Nunn, J.R. (1960) Biochem. J. 75, 557

* Wintersberger, E (1964). Hoppe- Seylers Z.Physiol. Chem. 336, 285

Wintersberger, E (1966). Biochem. biophys. Res. Commun. 25, 1

* Woodhouse, D.L. (1954a) Brit J. Cancer, 8, 346

* Woodhouse, D.L. (1954b) Brit. J. Cancer. 9, 418

* Yamagiwa, K. and Ichikawa, K. (1918). J. Cancer Res. 3, 1